PLANT PHYSIOLOGY

VOLUME 35 MAY, 1960 NUMBER 3

NET ASSIMILATION RATE AND GROWTH BEHAVIOR OF BEANS AS AFFECTED BY GIBBERELLIC ACID UREA AND SUGAR SPRAYS [1]

PAULO DE T. ALVIM

INTER-AMERICAN INSTITUTE OF AGRICULTURAL SCIENCES, ANDEAN ZONE, LIMA, PERU

Many workers have demonstrated the remarkable effect of gibberellins in producing increased rates of growth in size and weight. Weight increases usually are a result of higher photosynthetic activity, but a recent study with radioactive carbon (2) indicates that gibberellic acid did not enhance the rate of CO_2 fixation per unit of leaf tissue and did not alter the general pathway of the newly fixed carbon in the sugars, organic acid, and amino acid products.

The main purpose of the present study was to determine the influence of gibberellic acid on the net assimilation rate of a plant, as measured by the technique more commonly used by the English physiologists and lately reviewed by Watson (3). It is believed that this simple technique gives more reliable information on the average rate of photosynthesis than do those involving gas exchange measurements in which the plants or leaves are placed in an artificial environment. Sugar and urea sprays were included in the experiment in an attempt to determine whether or not carbohydrates and nitrogen might become limiting factors for growth when gibberellic acid is applied.

MATERIALS AND METHODS

Matched pairs of two-week-old red kidney bean seedlings (*Phaseolus vulgaris*) carefully selected for uniformity in size and leaf area and grown in Hoagland's nutrient solution, were placed in forty-eight 500 ml amber bottles, in eight groups of six, corresponding to the following treatments: 1) Control (no treatment). 2) Sucrose spray (10 % solution). 3) Urea spray (2 % solution). 4) Gibberellic acid spray (50 ppm solution). 5) Sucrose plus urea (10 % and 2 %, respectively). 6) Sucrose plus gibberellic acid (10 % and 50 ppm, respectively). 7) Urea plus gibberellic acid (2 % and 50 ppm, respectively). 8) Sucrose plus urea plus gibberellic acid (10 %, 2 % and 50 ppm, respectively).

Before the treatments were applied one seedling of each pair, taken at random, was used for dry weight and leaf area measurements. The remaining plants were then treated with the chemicals and distributed at random on the greenhouse bench. Sugar and urea sprays were repeated on the third and sixth day after the initial application. The plants were harvested on the tenth day for computing final dry weight (leaves, stem, roots, and total), plant height, and leaf area. Net assimilation rate (in grams per square decimeter of leaf surface and per day) was calculated by the formula (3).

$$NAR = \frac{(W_2-W_1)}{(L_2-L_1)} \frac{(\log_e L_2-\log_e L_1)}{(t_2-t_1)}$$

where W_1 and W_2 are total dry weight and L_1 and L_2 total leaf area at times t_1 and t_2, respectively. The relative growth rate (in grams per gram per day) was given by:

$$RGR = \frac{(\log_e W_2-\log_e W_1)}{(t_2-t_1)}$$

The data were analyzed for main effects and interactions of second and third order between gibberellic acid, sugar and urea, as a 2^3 factorial experiment.

RESULTS AND CONCLUSIONS

From the results presented in table I, which gives also the F values of statistical analysis, the following conclusions are derived:

1) Net assimilation rate (NAR) and relative growth rate (RGR) were both increased by gibberellic acid; in other words, the amount of dry matter produced by photosynthesis per unit of leaf area was significantly higher in plants receiving gibberellic acid. On a percentage basis, the average increase was 17.1 % for NAR (0.041 and 0.035 g/dm²/day,

[1] Received December 13, 1957.

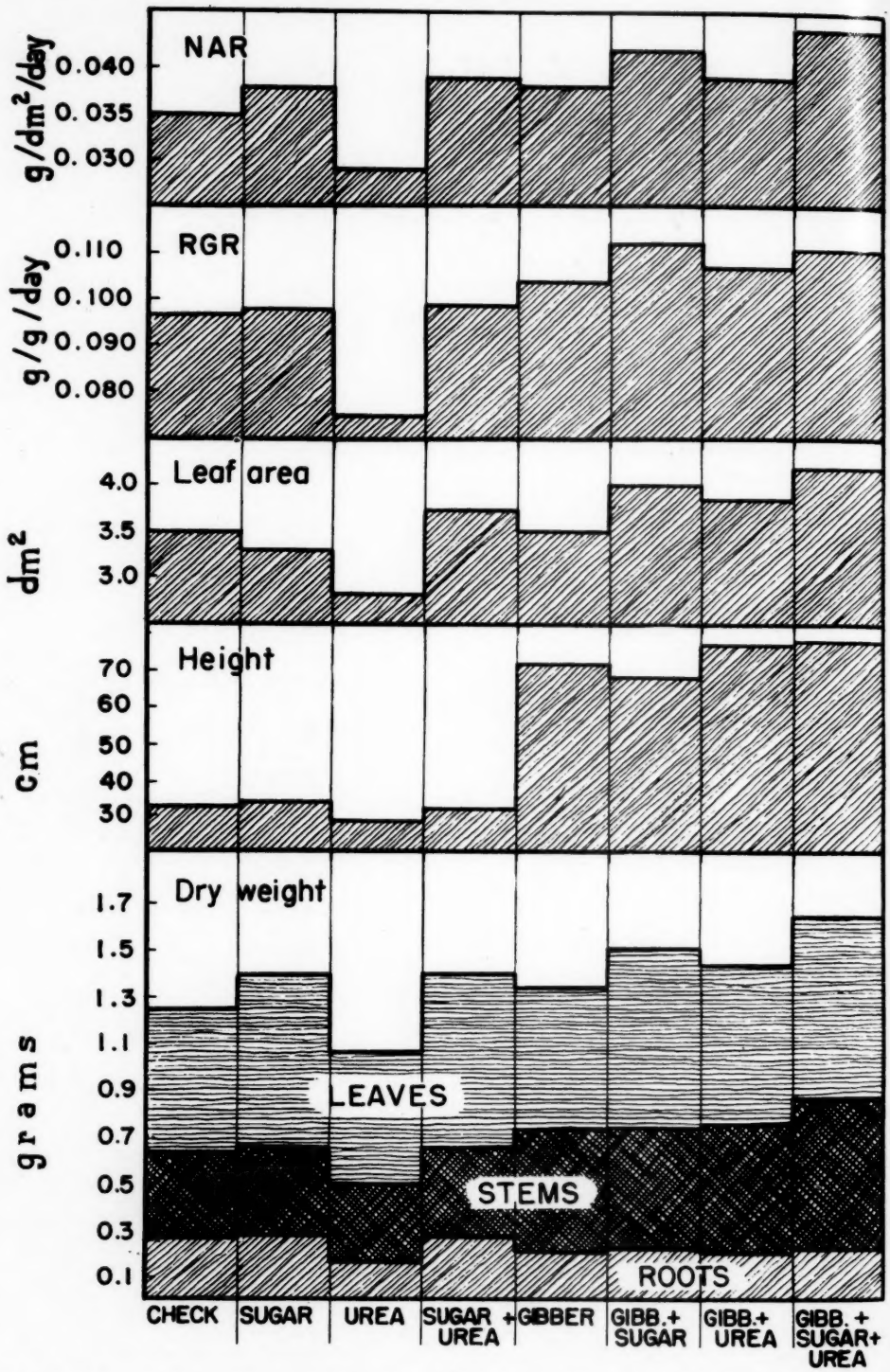


FIG. 1. Influence on the growth of red kidney bean plants of the treatments indicated. For explanation see text.

TABLE I

EFFECT OF GIBBERELLIC ACID, SUGAR, AND UREA SPRAYS ON NET ASSIMILATION RATE (NAR), RELATIVE GROWTH RATE (RGR), HEIGHT, LEAF AREA, AND DRY WEIGHT OF BEANS

TREATMENTS	NAR (G/DM²/D)	RGR (G/G/D)	HEIGHT (CM)	LEAF AREA (DM²)	DRY WEIGHT (G)			
					TOTAL	ROOTS	LEAF	STEM*
Check	0.035	0.097	32.1	3.53	1.25	0.279	0.628	0.347
Sugar	0.038	0.098	32.6	3.31	1.36	0.285	0.699	0.376
Urea	0.029	0.074	28.9	2.89	1.07	0.179	0.573	0.314
Sugar + Urea	0.039	0.098	31.2	3.74	1.41	0.274	0.754	0.385
Gibb. Acid	0.038	0.104	72.3	3.47	1.35	0.206	0.612	0.530
Gibb. + Sugar	0.042	0.112	69.9	3.99	1.52	0.222	0.778	0.519
Gibb. + Urea	0.039	0.107	79.5	3.85	1.45	0.209	0.692	0.546
Gibb. + Sugar + Urea	0.044	0.111	79.6	4.21	1.66	0.225	0.795	0.638
Sources of variation	*F values of statistical analysis*							
Effect of Gibb. acid	13.91***	16.76***	892.3***	7.06**	12.07***	6.68**	2.93	77.22***
Effect of Sugar	12.13***	5.06**	0.09	3.76	10.88***	5.01**	16.00***	2.22
Effect of Urea	0.13	1.48	4.41**	0.25	0.17	3.09	0.57	1.45
Interaction Gibb. x Sugar	0.49	0.49	0.73	0.10	0.07	1.33	0.01	0.41
Interaction Gibb. x Urea	2.06	2.36	13.12***	1.10	2.17	3.88	0.55	2.94
Interaction Sugar x Urea	1.92	1.16	0.55	1.38	1.25	2.18	0.13	2.52
Inter. Gibb. x Sugar x Urea	0.22	2.94	0.10	2.80	0.62	2.15	1.77	2.00

* Includes the petioles
** Significant (5 % level)
*** Highly significant (1 % level)

for treated and untreated plants, respectively) and 17.4 % for RGR (0.108 and 0.092 g/g/day for treated and untreated plants, respectively). Sugar also seemed to have increased NAR and RGR but this was obviously a result of the increase in dry weight brought about by the weight of the added sugar itself. Urea spray when applied alone caused necrotic spots on the leaves and decreased NAR and RGR, but in combination with sugar it seemed to stimulate NAR, although this effect was not statistically significant.

2) Plant height, as expected, showed the most striking response to gibberellic acid, with an average increase of 141.3 % (75.3 and 31.2 cm for treated and untreated plants, respectively). Urea sprays also decreased plant height when applied alone but had a stimulative effect when applied with gibberellic acid (positive interaction). This indicates that nitrogen might become a limiting factor for stem elongation when gibberellic acid is applied. Sugar sprays had no significant effect on plant height.

3) Leaf area increased 15.1 % by gibberellic acid (3.88 and 3.37 dm² for treated and untreated plants, respectively). Sugar sprays also seemed to have increased leaf area but this effect was not statistically significant. Urea sprays also reduced leaf area when applied alone (a 2 % solution, as used, is generally considered too strong for herbaceous plants), but not in combination with either sugar or gibberellic acid. Necrotic spots were not observed in plants treated with urea combined with either sugar of gibberellic acid, thus indicating that these substances are equally effective in preventing urea toxicity. Sugar sprays have been reported to have such an effect in other plants (1).

4) Total dry weight also was increased by gibberellic acid (17.3 %) and by sugar (16.4 %). Attention is called to the fact that the increase induced by the former took place in the stem whereas sugar sprays affected primarily leaf and, to a less extent, root dry weight. Gibberellic acid reduced root dry weight (17.9 %), a response that was effectively counteracted by sugar sprays (15.3 % increase). Urea sprays showed no significant influence on dry weight.

The above findings clearly indicate that gibberellic acid (probably as a consequence of its market influence on stem elongation) accelerated mobilization of photosynthates from the leaves to the stem. Higher NAR in plants receiving gibberellic acid probably resulted from this more rapid removal of photosynthates from the leaves. Carbohydrate supply limited root growth when gibberellic acid was applied, due to competition by the stem.

The above results do not support Haber and Tolbert's findings (1) according to which gibberellic acid does not affect the rate of photosynthesis. These authors worked with detached leaves, where mobilization of photosynthates is impeded and photosynthesis is bound to be retarded by the accumulation of its own products. Inasmuch as increases in NAR as induced by gibberellic acid seem to result from speeded translocation of photosynthates to the stem, results with detached leaves, in the author's opinion, are not applicable to intact plants.

Summary

Gibberellic acid increased net assimilation rate, relative growth rate, stem dry weight, leaf area, and plant height. Root dry weight was reduced and leaf dry weight was not significantly altered. Reduction in root dry weight induced by gibberellic acid could be effectively controlled by 10% sucrose sprays. Increase in net assimilation rate (photosynthesis) caused by gibberellic acid seemed to result from a more rapid translocation of photosynthates from the leaves to the stem. Urea sprays increased stem elongation of plants receiving gibberellic acid. Both sugar and gibberellic acid were effective in protecting against injury by 2% urea spray.

Literature Cited

1. Emmert, E. H. and J. E. Klinker. 1950. Spraying tomato foliage with sucrose to increase carbohydrates and protect against injury by urea sprays. Kentucky Agr. Exp. Sta. Bull. 550.
2. Haber, A. H. and N. E. Tolbert. 1957. Photosynthesis in gibberellin-treated leaves. Plant Physiol. 32: 152–153.
3. Watson, D. G. 1952. The physiological basis of variation in yield. Advances in Agron. 4: 101–145.

CONDITIONS AFFECTING ENZYME SYNTHESIS IN COTYLEDONS OF GERMINATING SEEDS [1, 2]

J. LOWELL YOUNG,[3] R. C. HUANG,[4] STEVE VANECKO,[5] JOY D. MARKS, AND J. E. VARNER

Department of Agricultural Biochemistry, The Ohio State University, Columbus

The rapid and manyfold increase in enzymatic activities of a phosphatase and an amylase in the cotyledons of germinating peas has been reported to be the result of net enzyme synthesis (8, 9). This conclusion was based upon the following findings:

I. Increases in these enzymatic activities are prevented by the protein synthesis inhibitors, chloramphenicol, and *p*-fluorophenylalanine.

II. Active mitochondria can be isolated from germinating pea cotyledons through the tenth day of germination. There is, therefore, presumably sufficient energy available for protein synthesis.

III. The microsomal fraction of pea cotyledons is capable of an in vivo and in vitro incorporation of glycine-C-14 into protein through the tenth day of germination.

IV. It has been shown in in vitro experiments that the microsomal fraction isolated from germinating peas is capable of a net synthesis of soluble proteins (7).

Further experimentation has shown that development of these enzymatic activities is dependent upon presence of the axis tissue (10). When the axis tissue is dissected away from the cotyledons of the dry seeds and the cotyledons placed under germination conditions, there is no development of phosphatase. These excised cotyledons rapidly lose mitochondrial activity; supposedly the loss of mitochondrial function is responsible for lack of enzyme synthesis in the excised cotyledons. Development of phosphatase activity in the cotyledons of germinating pea seeds is also greatly affected by temperature. At 16° C. although there is germination and slow growth in the seedling, there is no increase in the phosphatase activity of the cotyledons. During early stages of germination there is a rapid production in the axis tissue of a phosphatase apparently identical to the cotyledons phosphatase. Patterns of phosphatase activity in the cotyledons of germinating wax beans and the endosperm of castor beans are entirely different from the pattern in germinating peas.

Materials and Methods

Seeds were germinated in moist Vermiculite at temperatures indicated in each experiment. After the germination period the seedlings were washed free of Vermiculite and the different parts of the seedling separately homogenized in Tris buffer (0.1 M, pH 7.3) at 0° C with a Servall Omnimix. Fibrous material was removed by straining through cheese cloth. This extract was centrifuged at 20,000 × G for 1 hour and the supernatant fraction analyzed. Protein was determined by the method of Warburg and Christian (5). Phosphatase activity was meas-

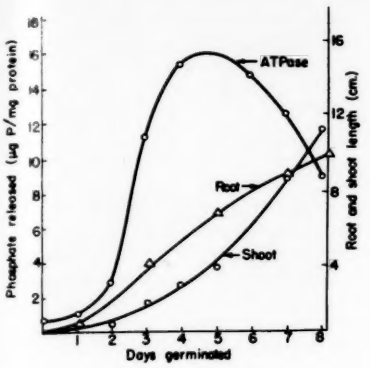
[1] Received for publication June 15, 1959.

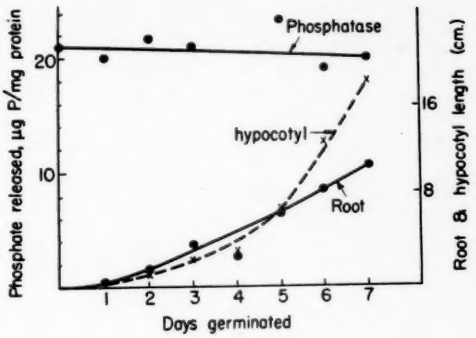
[2] Supported in part by grants from the Rockefeller Foundation, the National Institutes of Health, and The Development Fund of The Ohio State University.

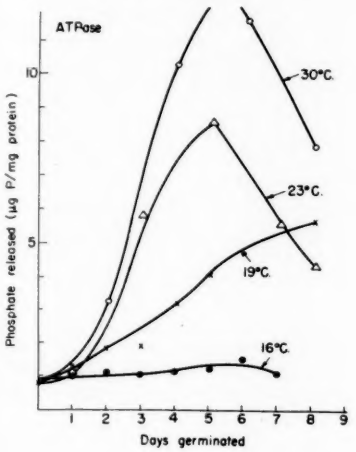
[3] Present address: Oregon State College, Corvallis.

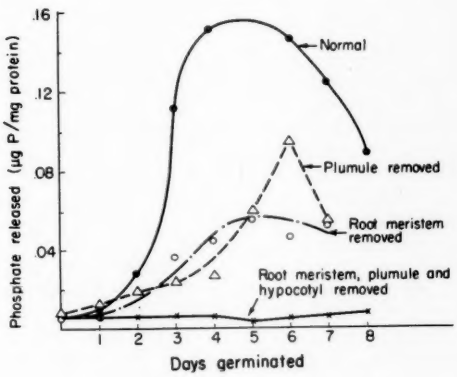
[4] Charles F. Kettering Foundation Predoctoral Fellow.

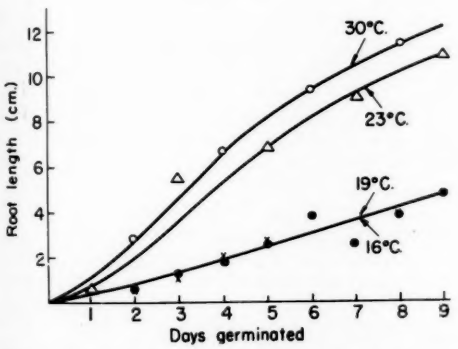
[5] Present address: Marquette University, Milwaukee, Wis.











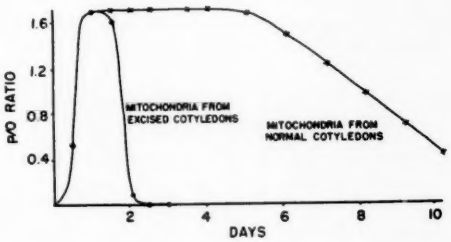


FIG. 1 (*upper left*). ATP-ase activity in cotyledons of germinating pea seeds. The 20,000 × G supernatant fraction of the cotyledon extract was used for the enzyme assay.

FIG. 2 (*middle left*). ATP-ase activity in cotyledons of germinating peas as a function of temperature of germination. The 20,000 × G supernatant fraction of the cotyledon extract was used for the enzyme assay.

FIG. 3 (*lower left*). Growth of germinating peas as a function of temperature of germination.

FIG. 4 (*upper right*). Phosphatase activity in cotyledons of germinating black wax-bean seeds. The 20,000 × G supernatant fraction of cotyledon extract was used for the enzyme assay.

FIG. 5 (*middle right*). Influence of growing tissues on development of pea cotyledon phosphatase. The 20,000 × G supernatant fraction of the cotyledon extract was used for the enzyme assay.

FIG. 6 (*lower right*). P/O ratios of mitochondria from normal and excised pea cotyledons.

ured by incubating 1.0 ml of the extract with 0.033 M Tris[6] (pH 7.1), 0.001 M $MgCl_2$ and 0.004 M ATP in a total volume of 3.0 ml at 38° C for 15 minutes. The reaction was stopped by adding 1.0 ml of TCA and the phosphate released determined (3). With these assay conditions, the quantity of phosphate released was a linear function of time and enzyme concentration over the range of times and concentrations used.

Mitochondria were isolated by a procedure similar to that of Millerd et al (1). Ten grams of cotyledons was cooled to 0° C in a mortar with an equal weight of sand. The cotyledons were ground in 10 to 15 ml of 0.45 M sucrose and 0.05 M phosphate (pH 7.1) and the resulting slurry diluted to 40 ml with cold sucrose-phosphate solution, filtered through cheese cloth, and the filtrate centrifuged at 1,500 × G for 5 minutes. The supernatant fraction was centrifuged at 15,000 × G for 15 minutes. This sediment was resuspended in 20 ml of cold sucrose-phosphate and again centrifuged at 15,000 × G for 15 minutes. The sediment was taken up in 5 ml of 0.5 M sucrose and aliquots taken for assay. Oxygen uptake by the isolated mitochondria was measured in a conventional Warburg respirometer at 30° C. The reaction was stopped with TCA, and inorganic phosphate determined by the method of Sumner (3). The cotyledon extract, or dialysate was prepared by extracting the cotyledons of four-day-old germinating peas with an equal weight of 0.02 M phosphate solution (pH 7.1). This slurry was centrifuged at 15,000 × G for 15 minutes and the supernatant fraction dialyzed against an equal volume of 0.02 M phosphate solution (pH 7.1) at 0° C for 24 hours.

The measurement of the in vivo incorporation of glycine-C-14 into the protein of the cotyledons was as described by Siekevitz (2). The samples were assayed for radioactivity with a Nuclear model D-47 gas flow detector and standard scaling circuit after being uniformly deposited on glass planchets. The in vitro incorporation of glycine-C-14 into the protein of the ribonucleoprotein particles was measured as described by Webster (6).

Results and Discussion

In the cotyledons of germinating peas (var. Alaska), there is a several-fold increase in phosphatase activity (fig 1) which is rather specific for ATP and ADP (these two substrates are hydrolyzed at about 10 to 30 times the rate of any one of six other substrates tested), has a pH optimum of 7.1 and requires a divalent cation (8, 9). The rate of development of this phosphatase activity is greatly influenced by the temperature of germination (fig 2). Although there was a six-fold increase in the cotyledon phosphatase in 8 days at 19° C, there was no appreciable increase at 16° C. The rate of growth of the seedling as measured by elongation of the root was the same at 19° and 16° C (fig 3). It is not possible to assign a metabolic function to this phosphatase at this time. It seems clear, however, that at temperatures below 19° C its function is not a limiting one in the growth of the seedling. The problem of assigning a function is not decreased by the observation that phosphatase in the cotyledons of wax beans not only does not increase during germination (fig 4) but also seems to be a different enzyme in that it is inhibited to a much greater extent by phosphate than is the pea phosphatase. In castor bean endosperm the initial level of phosphatase activity was low (2 μg P released/mg protein/15 min at 38° C) and it had only doubled by the seventh day of germination. Although generalization is not possible on the basis of results from these examples, it may be that the rapid development of phosphatase activity in the cotyledons of germinating peas is unique rather than representative, and that such activity is associated with the known deletrious effects of high temperatures on pea seedlings. It will be of great interest to learn whether the pattern of development of this phosphatase is the same in temperature-susceptible and temperature-resistant peas, and whether the increased phosphatase activity results in a decreased cellular level of ATP. Likewise, it would be interesting to determine the effect of adenine on the ATP level in both kinds of peas.

Extracts of the root and stem of germinating peas also contain a phosphatase apparently identical to the cotyledon phosphatase. The distribution of phosphatase activity (with ATP as substrate) in the seedling is shown in table I. The level and distribution of axis tissue phosphatase as influenced by germination temperature has not been studied.

The presence of an ATP-ADP hydrolase in the meristematic regions where there is the greatest demand for energy for synthesis of cell-stuff seems illogical. It is possible that this enzyme functions in vivo as a transphosphorylase. The enzyme has been purified 200-fold, but so far no transfer activity has been found.

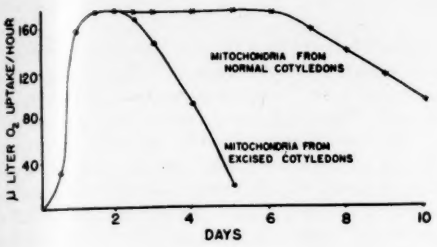
Table I

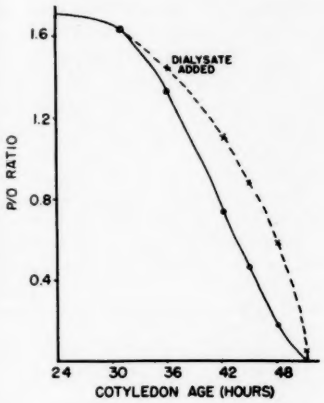
Distribution of ATPase in Various Sections of 4-day-old Germinating Pea Seedling

Extract* from seedling part	Relative activity μg P released/mg protein
Tip cm of root	40
2nd cm of root	26
3rd cm of root	31
Hypocotyl tissue	17
2nd cm of stem	42
Tip cm of stem	72
Cotyledons	6
Whole seedling	17

* Activity was measured in supernatant solutions obtained by centrifuging homogenized plant tissues at 20,000 × G for one hr.

[6] Abbreviations used; Tris (tris (hydroxymethyl)-aminomethane); ATP, (adenosine triphosphate); TCA (trichloracetic acid), ADP (adenosine diphosphate).





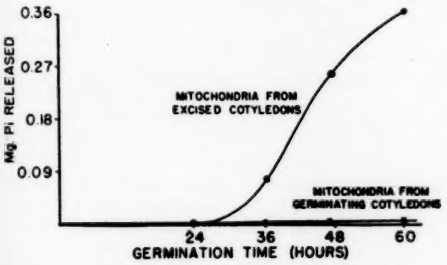


FIG. 7 (*top*). Oxygen uptake of mitochondria from normal and excised pea cotyledons.

FIG. 8 (*center*). Effect of dialysate on P/O ratio of mitochondria from excised pea cotyledons.

FIG. 9 (*bottom*). ATP-ase activity of mitochondria isolated from pea cotyledons.

A striking feature of the development of the pea cotyledons phosphatase is its dependence upon the presence of some portion of the axis tissue (fig 5). This is presumably related to the fact that the cotyledon mitochondria lose function (and thereby the energy source for protein synthesis is lost) when excised cotyledons are incubated under germination conditions (4).

Mitochondria isolated from two-day-old excised cotyledons (fig 6) have no phosphorylative ability while mitochondria isolated from cotyledons of normally germinating peas still have some phosphorylative ability after ten days of germination. Mitochondrial fractions from excised cotyledons also lose their capacity for oxygen uptake (fig 7). The fact that there is no increase in oxygen uptake by the mitochondria at the time the P/O ratio begins decreasing (figs 6, 7) suggests that the mitochondria are already performing electron transport function at full capacity. The data in Table II indicate that the mitochondria isolated from the cotyledons of normally germinating peas are loosely coupled. That is, there was no decrease in oxygen uptake in the absence of added ADP although phosphorylation decreased to one-ninth the rate in the presence of ADP. These mitochondria will also oxidize DPNH and *a*-ketoglutarate with P/O ratios of 1.5 to 1.9, and 2.6 to 3.0, respectively. D,L-β-hydroxybutyrate was not oxidized.

Loss of function of mitochondria isolated from the excised cotyledons is partially reversible in vitro as is shown by the effect of added dialysate from the supernatant fraction of cotyledon extract (fig 8). Presumably this loss of mitochondrial activity is due to a leakage of soluble cofactors from the mitochondria although components of the dialysate have not been identified. An ATP-ase activity localized in the mitochondria of the excised cotyledons increases rapidly after about 30 hrs (fig 9). Undoubtedly this is a manifestation of the degradation of the phosphorylative function of the mitochondria and should not be confused with the phosphatase which appears in the supernatant fraction of the cotyledons of germinating peas (fig 1). There is no indication of the nature of the substance from the axis tissue which

TABLE II

REQUIREMENTS FOR OXIDATION OF SUCCINATE AND COUPLED PHOSPHORYLATION BY MITOCHONDRIA FROM COTYLEDONS OF 2-DAY-OLD GERMINATING PEAS

SYSTEM	μl O_2 UPTAKE/HR	P/O RATIO
Complete	140*	1.7*
Complete — succinate	21*	...
Complete — ADP	147*	0.2*
Complete — Mg^{++}	158*	1.3*
Complete — hexokinase	153*	0.9*
Complete + DNP (1×10^{-4} M)	122*	0*

* Average of two determinations

Complete system contained 0.02 M succinate, 0.2 M sucrose, 0.1 M glucose, 0.01 M phosphate, 0.01 M NaF, 0.001 M ADP, 0.004 M Mg^{++}, 0.1 Mg hexokinase and 1 ml of the mitochondrial suspension (1 mg of nitrogen).

TABLE III

IN VIVO AND IN VITRO INCORPORATION OF GLYCINE-C-14 INTO MICROSOMAL PROTEIN

COTYLEDON AGE (DAYS)	IN VIVO INCORPORATION	IN VITRO INCORPORATION
Cotyledons of Germinating Peas		
1	370 cpm	75
2	300	77
4	100	73
6	88	72
9	88	85
15	9	97
Excised pea cotyledons		
1	550	73
3	0	77

enables the maintenance of mitochondrial function, or whether there may be additional factors controlling other processes. The problem of the identity of this factor or factors is being studied in lima beans where the effect of excision of axis tissue develops earlier and is more readily observable. These observations are of more than passing interest because they indicate that in pea cotyledons the synthetic events (formation of phosphatase, amylase, etc.) which precede and bring about the orderly degradation of this storage tissue are controlled by some means by the growing portions of the seedling.

Further evidence that the cotyledons are capable of synthetic activity is shown in table III. Perhaps the most interesting feature of these data is the observation that the microsomal capacity for in vitro incorporation of labeled amino acids persists beyond that time in the age of the cotyledons that the mitochondria are functional. This immediately suggests that the aging of cotyledon cells might be due to a loss of energy required for the performance of necessary work functions rather than to loss of enzymes performing these work functions.

SUMMARY

It has been shown that the functional integrity of the mitochondria in the cotyledons of germinating peas is maintained only when axis tissues remain attached to the cotyledons. When excised pea cotyledons are incubated under germination conditions, there is a rapid loss of mitochondrial function. Also the cotyledon phosphatase activity which increases during germination fails to increase during incubation of the excised cotyledons. This is presumed to result from the loss of energy source required for protein synthesis. These observations indicate that the events which occur in cotyledons of germinating seeds are under the control of a factor or factors from the axis tissue.

LITERATURE CITED

1. MILLERD, A., J. BONNER, B. AXELROD, and R. S. BANDURSKI. 1951. Oxidative and phosphorylation activity of plant mitochondria. Proc. Nat. Acad. Sci. 37: 855.
2. SIEKEVITZ, P. 1952. Uptake of radioactive alanine in vitro into the protein of rat liver fractions. Jour. Biol. Chem. 195: 549.
3. SUMNER, J. B. 1944. A method for the colorimetric determination of phosphorus. Science 100: 413.
4. VANECKO, STEVE. 1957. Oxidative phosphorylation in cotyledons of *Pisum sativum*, var. Alaska. Ph.D. Dissertation, Ohio State University.
5. WARBURG, O. and W. CHRISTIAN. 1941. Isolierung and Kristallisation des Garungsferment Enolase. Biochem. Zeits. 310: 384.
6. WEBSTER, G. C. 1957. Amino acid incorporation by intact and disrupted ribonucleoprotein particles. Jour. Biol. Chem.: 229, 535.
7. WEBSTER, G. C. 1959. Studies on the mechanism of protein synthesis by isolated nucleoprotein particles. Federation Proc., 18: 348.
8. YOUNG, J. LOWELL and J. E. VARNER. 1957. Enzyme synthesis in the cotyledons of germinating seeds. Federation Proceedings 16: 275.
9. YOUNG, J. LOWELL and J. E. VARNER. Enzyme synthesis in the cotyledons of germinating seeds. Arch. Biochem. Biophys. (In press).
10. YOUNG, J. LOWELL. 1957. Conditions affecting enzyme synthesis in the cotyledons of germinating seeds. Plant Physiol. 32 suppl: xxv.

PHYSIOLOGICAL EFFECTS OF GIBBERELLIC ACID: I. ON CARBOHYDRATE METABOLISM AND AMYLASE ACTIVITY OF BARLEY ENDOSPERM[1]

LESLIE G. PALEG

WAITE AGRICULTURAL RESEARCH INSTITUTE, ADELAIDE, S. A., AUSTRALIA

Research into the physiological effects of gibberellic acid (GA) and other gibberellins has been confined largely to descriptive accounts of stimulatory (3) and inhibitory (18) actions, while few studies have attempted to integrate the action of GA with the normal physiological processes of the plant. One of the most clearly recognized stimulatory actions of GA is its ability to break dormancy (3), or conversely, its germination-promoting effect. Hayashi (7) demonstrated that treatment with gibberellins hastened the germination of barley and rice and, more recently, other workers have reported similar successes also with barley (8, 12, 19). A hastening of germination, as described by Hayashi, almost necessarily involves the increased breakdown of carbohydrate reserves in the endosperm, and although this tissue is well classified with respect to its carbohydrate, amino acid, and protein constituents, little is known about how its metabolism is controlled enzymatically.

A recurring suggestion in the literature is that enzymatic activity of the endosperm is derived by secretion of enzymes into that tissue by the embryo. Kirsop and Pollack (8) have demonstrated that there is, in fact, a positive correlation between the presence of the embryo and the β-amylase content of the endosperm, with amylolytic action falling off sharply following removal of the embryo. In 1947, however, Engel (5) showed that most of the β-amylase in the barley grain was in the endosperm, so it is unlikely that β-amylase secretion by the embryo plays a decisive role in the carbohydrate metabolism of the endosperm. The results of Kirsop and Pollack, then, may be interpreted by postulating the secretion of some substance other than β-amylase into the endosperm by the embryo. It is noteworthy, with respect to the distribution of this enzyme in the grain, that Hayashi (7) also demonstrated that gibberellins increase the extractable β-amylase of barley grains during germination without affecting the activity of the enzyme. One might propose the increase in β-amylase concentration in the endosperm found during germination as being due to an embryonal gibberellin, but Pollack (19) has reported that the effect of GA is limited to the embryo.

There is little evidence that amylase is involved at all in the breakdown of native starch in vivo, and Whelan (25) has expressed doubts about its importance in this process. Amylase activity in barley increases, however, as germination progresses, and it is enhanced by compounds which stimulate germination. In addition, maltose is a compound commonly found in germinating grain and, with the exception of D-enzyme, the amylases are the only enzymes in higher plants known to be capable of its formation. It is difficult to conceive of starch hydrolysis in the endosperm taking place in the absence of amylase activity, and any indication of the release of sugar coincident with, and possibly even dependent upon amylase activation would be useful in estimating the endogenous role of this enzyme.

MATERIALS AND METHODS

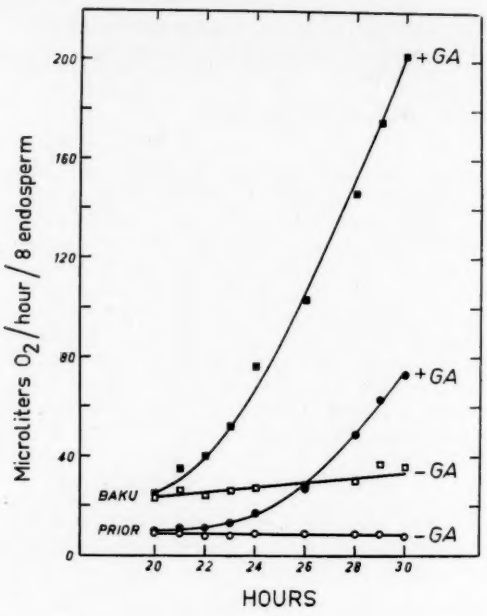
All water used in these experiments was doubly glass distilled, and all GA solutions were freshly prepared the day of the experiment.

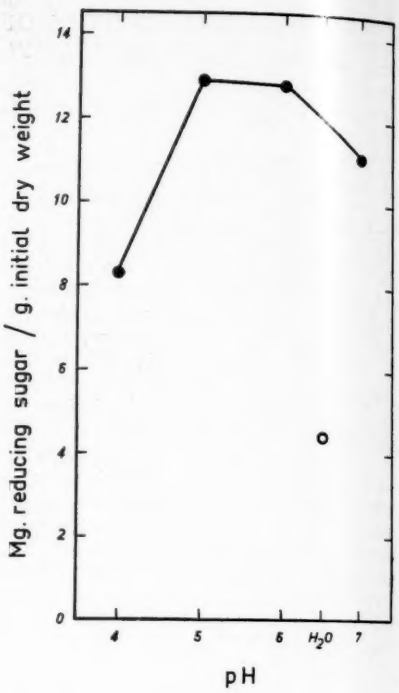
RESPIRATION. *Hordeum distichum* var. Baku (a naked seed) and Prior (a husked seed) from the 1956 harvest were used. The husk of the Prior seeds was removed by hand and seeds of both varieties were cut in half transversely before treatment was commenced. The endosperm halves were incubated in the dark at 25° C for 18 hours. Petri dishes (4 in) containing eight endosperm halves and two sheets of filter paper, moistened with either 8 ml water or 8 ml water plus 200 gammas GA, were used. Following incubation, each sample of endosperm was rinsed thoroughly with water, blotted and weighed. The samples were then placed in Warburg flasks containing solutions similar to those of the initial soaking period (i.e., either 2 ml water or 2 ml water plus 200 gammas GA). The center wells contained 0.2 ml 10 % NaOH and the temperature was maintained at 25° C.

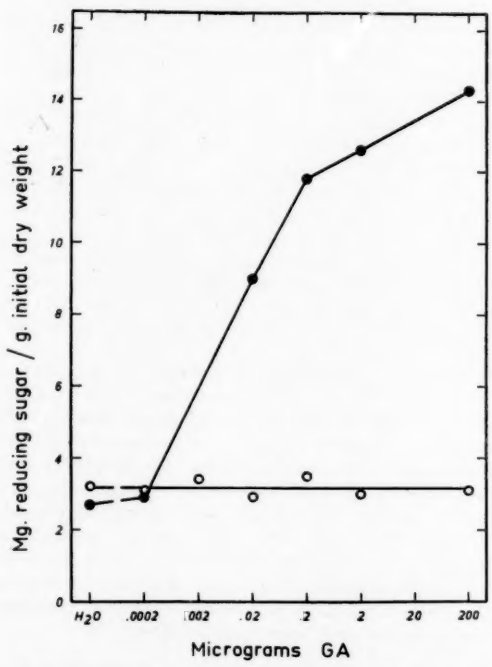
STORAGE CONDITIONS. As noted by Merry and Goddard (10), seeds with a constant initial moisture content evidence much less variability than seeds with differing moisture contents. Consequently, Merry and Goddard's storage technique was adopted for all seeds other than those used in the respiration experiments. A saturated $CaCl_2$ solution containing crystals of undissolved $CaCl_2$ was placed in the bottom of a desiccator and seeds of variety Prior from the 1957 harvest were equilibrated over the $CaCl_2$ for several days before use. In this way, the initial moisture content of the seeds was maintained at 8.3 %.

SUGAR ASSAY. One ml aliquots of the media surrounding the endosperm were diluted to 10 ml with water and approximately 1 g of ion-exchange resin (Amberlite IR-120H) was added. The mixture was

[1] Received manuscript June 22, 1959.







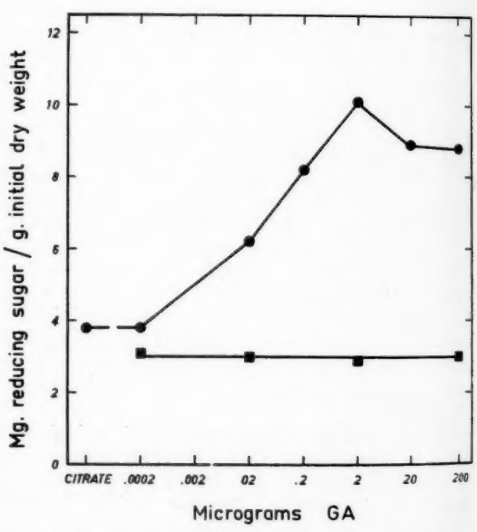


FIG. 1 (*upper left*). Effect of 200 μg GA/2 ml solution on respiration of Baku and Prior endosperm. (Weights of endosperm of both varieties following pretreatment was similar).

FIG. 2 (*lower left*). Effect of GA on sugar release by sterilized Prior endosperm: ●, normal endosperm; ○, endosperm heated at 100° C for 1 hour before GA treatment. Total volume = 3 ml solution.

shaken 15 times in 15 minutes after which it was filtered through one thickness of Whatman No. 1 filter paper. Two 1 ml aliquots of the filtrate were assayed for reducing sugars with Somogyi's 1952 alkaline copper reagent (23). The reagent consists of 24 g anhydrous sodium carbonate, 12 g sodium potassium tartrate, 16 g sodium bicarbonate, 4 g copper sulfate ($5H_2O$), and 180 g anhydrous sodium sulfate in 1 l of solution.

One ml of this reagent was mixed in a test tube with the 1 ml aliquot. Test tubes were covered with marbles and heated in a vigorously boiling water bath for exactly 10 minutes. They were then cooled for 5 minutes in cold water and 1 ml of Nelson's arsenomolybdate reagent (15) was added. The resultant solutions were again mixed, diluted to 10 ml and read at 560 mμ in a Unicam SP 350 spectrophotometer. All concentrations of citrate buffer used in the experiments were low enough to avoid interference in the reducing sugar assay (17).

Chromatography. 50 μl aliquots of the various media surrounding the endosperm were applied as spots to a sheet of Whatman No. 1 chromatography paper along with control spots. The sheet was equilibrated overnight at 25° C over the organic phase of a 1-butanol : water : acetic acid (v/v 4 : 5 : 1) mixture, and developed downward with a similar mixture. The chromatogram was removed after about 28 hours, dried for a half hour, and several of the control strips were cut out. These were sprayed with benzidine-trichloracetic acid (0.25 g of benzidine, 5 ml glacial acetic acid, 5 ml 40 % trichloracetic acid, and 40 ml ethanol) and the positions of glucose and maltose on the test strips corresponding to these sugars on the control strips, were cut out and eluted for 1 hour with 2 ml water. The eluates were then assayed for sugars as indicated above.

Amylase Activity. Amylase activity in the medium surrounding the endosperm was measured by incubating an aliquot of the medium with soluble starch in 0.005 M acetate buffer (pH 4.6) at 30° C for 20 minutes. The reaction was terminated with $HgCl_2$ and an aliquot of the solution was assayed for maltose. Amounts of sugar present in the enzyme and starch solutions were subtracted from the final values.

Results and Discussion

The endosperm has been recognized for some time as a tissue which is essentially devoid of respiratory activity, and this has been explained by Barnell (2) as being due to a shortage of respiratory sites. Stanley (24) has demonstrated, however, that the pine endosperm does contain respiratory enzymes and that the activity of the enzymes varies during germination. Since this study was undertaken to determine the possible influence of GA on carbohydrate metabolism, it was thought necessary to reinvestigate the respiratory activity of the barley endosperm. Figure 1 illustrates the oxygen uptake observed in Warburg flasks during treatment of barley endosperm with 200 gammas GA. When GA is present the endosperm of both varieties show evidence of a high rate of oxygen uptake, while in the absence of GA the observed respiration is slight. Since, however, the endosperm is normally believed to be lacking a sufficient number of respiratory centers to carry on respiration of this magnitude and since also, bacterial contamination of the barley grain is very prevalent, it became necessary to ascertain whether the observed activity was bacterial or endospermal in nature.

Dilutions of solutions from Warbug flasks of subsequent experiments were plated out with agar (containing 0.1 % peptone, 0.1 % Difco yeast extract, 0.1 % glucose, and tap water as a source of salts), and incubated at 30° C for 24 hours. It was possible to count bacterial colonies, each theoretically arising from a single cell in the original media, and in this way the active oxygen uptake was definitely associated with the presence of a very large number of an unidentified, rapidly reproducing strain of bacteria. In no case of respiratory activity were the bacteria absent, nor did GA stimulate the respiration of endosperm in the absence of bacteria. Since the flasks contained only 200 gamma of GA, however, it was apparent that the observed respiration could not be a function of the oxidation of the GA. Rather, it seemed more likely that GA was causing the production or liberation of something from the endosperm which enabled the bacteria to respire and reproduce readily.

The necessity for a sterilization procedure which would eliminate the influence of the bacteria was indicated, and the following technique was adopted:

I. Prior seeds with husks (initial moisture content of 8.3 %) were soaked for exactly 20 minutes in a 5 % calcium hypochlorite solution. (The solution was freshly prepared before each experiment by shaking the calcium hypochlorite with water for 10 minutes in a glass-stoppered flask and passing the resultant mixture through one thickness of Whatman No. 1 filter paper).

II. Seeds were rinsed three times with water and placed in a 4 in Petri dish containing one piece of filter paper and 3 ml water.

III. Husks were then removed by hand during the subsequent 40 minutes and peeled seeds were placed on similarly moistened filter paper in a second Petri dish.

Fig. 3 (*upper right*). Effect of pH on the sugar release induced by GA: ●, 20 μg GA; ○, H_2O. Total volume = 3 ml solution.

Fig. 4 (*lower right*). Effect of 0.01 M citrate (●), and acetate buffers (■) at pH 5.5 on GA-induced sugar release. Total volume = 3 ml solution.

IV. Seeds were returned to the calcium hypochlorite solution for a further sterilization period of 1 hour.

V. After decanting the calcium hypochlorite, the seeds were rinsed ten times with water and blotted. Endosperm were obtained by cutting the grain in half transversely and were weighed in groups of eight before being placed, at random, into small (2 in) Petri dishes containing 500 gammas streptomycin and the test solution, (*total volume*, 3 *ml*).

Embryos obtained from the above procedure germinated normally without a sign of bacterial contamination; bacterial counts similar to those indicated above showed that the test solutions, containing the endosperm, were substantially free from bacteria after 24 hours. Furthermore, because of the very short time (2 hrs) that the embryo was in contact with the endosperm following the start of the sterilization procedure and the fact that the grains were cut well behind the junction of embryo and endosperm, it is unlikely that the embryo was able to exert any influence over the subsequent reactions of the portion of the endosperm under investigation.

Prior seed were passed through the sterilization procedure outlined above and incubated at 24° C for 22 hours with varying amounts of GA. Each concentration of GA was tested in duplicate and, following treatment with the ion-exchange resin, duplicate aliquots were taken for the reducing sugar assay. The amounts of reducing sugar present in the media surrounding the endosperm are presented in figure 2 (closed circle curve) and are expressed as mg reducing sugar (as glucose) per g initial dry weight. Initial dry weights for figure 2, were determined by ignoring the moisture absorbed by the grain during the 2 hour sterilization treatment and subtracting 8.3 % from the weight of the endosperm, determined immediately before incubation.

From the closed circle curve of figure 2 it can be seen that, as a result of GA treatment, the endosperm does in fact liberate reducing sugar into the surrounding medium. Of particular interest is the range over which GA is effective, from about 2×10^{-4} μg to probably well over 2×10^{2} μg, or a factor of 1,000,000. Only one other instance has been reported [the effect of GA on dwarf peas (4)] in which GA has a range of effective concentrations approaching this figure.

To determine whether the release of sugar was a function of enzymatic activity, the previous experiment was duplicated, except that the grain was subjected to dry heat at 100° C for 1 hour in a still air oven before the sterilization procedure was begun. These results are also presented in figure 2 as the open circle curve. It should be pointed out that the initial moisture content of 8.3 %, on which the initial dry weight figures are based, was in this case slightly in error as a result of the heat treatment. Since a change in initial moisture of 3 %, however, would change the reducing sugar values by only 0.1 mg, it is unlikely that the error in moisture content would influence the final values appreciably. It may be noted that the effect of prior heat is to obliterate the sugar release induced by GA, indicating that an enzyme is most probably involved and that the effect of GA on sugar release is a secondary one.

To determine which sugars were being liberated, and in what amounts, an aliquot of the medium of one Petri dish at each GA concentration was chromatographed and eluted. Two monosaccharides, glucose and fructose, and two disaccharides, sucrose and maltose, were identified. In addition, there were two spots between maltose and the origin in the positions of maltotriose and maltotetrose although they were not further investigated.

Table I contains the values of eluted glucose and maltose determined by comparison with known amounts of glucose and maltose which also were passed through the same procedure. As the concentration of GA was increased, the amounts of these sugars present in the surrounding medium also increased. The combined amounts of glucose and maltose in table I exceed the values presented in figure 2. This is due to the fact that maltose was estimated as glucose in the calculation of reducing sugar content for figure 2 although it has less than half the reducing power of glucose. When the eluted values for maltose from table I were calculated as glucose it was determined that maltose accounts for about 15 %, and glucose about 60 % of the reducing sugar values shown in figure 2.

The carbohydrate composition of barley grain at maturity and during malting has been investigated several times with different varieties (6, 22). In each case, the almost complete absence of maltose in the ungerminated grain has been noted. In the present experiments, the levels of maltose attained in the medium surrounding the endosperm could not result from diffusion of existing maltose, but must be a function of the enzymic dissolution of starch. The results of heat treatment prior to exposure of the endosperm to GA substantiate this conclusion.

Although GA is not a strong acid, over a concentration range of this magnitude we do find changes in the pH of its water solutions. To determine whether

TABLE I

EFFECT OF GA ON GLUCOSE AND MALTOSE LIBERATION*

μg GA/3 ML SOLUTION	SUGAR CONCENTRATION	
	MALTOSE	GLUCOSE
	mg/g initial dry weight	
0(H_2O)	1.5	1.7
0.002	0.8	1.8
0.02	4.9	6.1
0.2	5.4	7.6
2.	3.6	7.3
200.	8.5	9.5

* Sugar values determined by chromatography and elution of 50 μl aliquots of solution surrounding endosperm.

the pH of the surrounding medium exerted any effect on the liberation of reducing sugars, endosperm were incubated in solutions cotaining 20 gammas GA which had previously been adjusted to pH 4, 5, 6, or 7 with acetic acid or sodium hydroxide (fig 3). For this experiment the actual moisture content of the endosperm, following the 2 hour sterilization procedure, was determined and found to be 11.6 % indicating an increase of 3.3 % over the moisture content produced by the storage conditions. This value was duplicated closely in other experiments and as a result, the calculation of all dry weights in this and subsequent experiments was based on a moisture content of 11.6 %. In actual fact, although the reducing sugar values are more precise, the absolute amounts are changed very little.

It is apparent that pH does influence the GA-induced sugar release, although results indicate that the reducing sugar values obtained are far more dependent upon the presence of GA than they are on the changes in pH caused by its varying concentrations. The pH of the water control was 6.5. A comparison of the shape of the sugar release curve at different pH levels with the pH-activity curve of β-amylase (13) indicates a close similarity, with both curves showing a maximum close to pH 5.5. Since the necessity for pH control was indicated, GA-induced reducing sugar release was determined in the presence of 0.01 M acetate and citrate buffers at pH 5.5 (fig 4). Citrate, the closed-circle curve, interfered with the sugar release, producing about a 25 % inhibition (compare with fig 2) particularly evident at the higher GA levels. Acetate, however, the closed-square curve, completely suppressed the sugar release promoted by GA.

To ascertain whether the acetate effect was due to the acetate buffer per se, or due to concentration of the buffer, a comparison of the effects of four buffers, phthalate, acetate, citrate, and maleate, at 0.001 M, pH 5.5, was made. Table II, in which the results are presented, indicates that the four buffers exerted no influence on the water-induced sugar release. In the presence of GA, however, the differential effects of the buffers were more evident. Phthalate inhibited the sugar release to about 50 % while the effect of acetate, although much reduced from what it was at 0.01 M (fig 4), was still sufficient to produce about a 30 % inhibition. The inhibition produced by citrate at 0.01 M was, similarly to the acetate effect, greatly alleviated by reducing the concentration to 0.001 M, while maleate seems to produce no inhibition at this concentration. It seemed, therefore, that the enzyme with which we were dealing was quite as sensitive to changes in buffer concentration as to changes in pH.

TABLE II

EFFECT OF 0.001 M BUFFERS ON GA-INDUCED SUGAR RELEASE

BUFFER	REDUCING SUGAR CONCENTRATION	
	H_2O	GA (20 μg/3 ML SOLUTION)
	mg/g initial dry weight	
None	2.5	
Phthalate	2.4	7.4
Acetate	2.8	10.6
Citrate	2.6	13.6
Maleate	2.4	14.1

In 1941 Ballou and Luck (1) reported the results of a series of experiments dealing with the activity of β-amylase at different pH levels and in different buffers. Their experiments were performed, mainly, at a constant ionic strength. If their curves for β-amylase activity in phthalate, acetate, and citrate buffers, at the acid side of the optimum (below pH 5, where the effects of the buffer anions are most evident) are compared, β-amylase is seen to be least active in phthalate, more active in acetate, and most active in citrate buffer. From Ballou and Luck's curves it may also be seen that increases in buffer concentrations (the means by which pH is varied at a constant ionic strength) result in marked diminutions in the activity of β-amylase.

Although the ionic strength of the buffers was not held constant in the present experiments, the similarity in effects of phthalate, acetate, and citrate buffers in Ballou and Luck's experiments and in the control of GA-induced sugar release, can be noted.

β-amylase has been found by Sandegren (21) to be closely related to the albumin protein fraction in the barley grain. As defined originally by Osborne (16), the albumin fraction is the only plant protein fraction that is soluble in water. With this in mind, then, one would expect increased amylase activity to be a result of either an activation of the enzyme by GA so that each molecule of enzyme produced more sugar from a starch substrate, or an increase in the number of active amylase molecules present. In either case, if amylase is responsible for our observed sugar release, we might reasonably expect to find some active amylase in solution in the medium surrounding the endosperm, because it is water soluble. If, then, GA were activating the amylase molecule, it should be possible, by adding GA and starch to an assay containing an aliquot of the solution surrounding the endosperm, to increase the amount of sugar produced as compared with that produced in an assay in the absence of added GA. On the other hand, if GA were causing an increase in the number of active amylase molecules, this should be reflected, since they are water soluble, by an increase in the amylase activity in the medium surrounding the endosperm, as compared with the activity of the medium in the absence of GA.

Endosperm were subjected to the usual sterilization procedure and duplicate Petri dishes, containing 500 gammas streptomycin and 0.001 M acetate buffer in the presence and absence of 200 gammas GA, were incubated in the dark for 22 hours at 24° C. The solutions surrounding the endosperm, in the two Petri dishes containing GA, were combined, as were the solutions from the two Petri dishes lacking GA.

TABLE III

EFFECT OF GA ON LIBERATION AND ACTIVITY OF β-AMYLASE DERIVED FROM ENDOSPERM

ENDOSPERM TREATMENT*	ASSAY TREATMENT**	MG REDUCING SUGAR PRODUCED IN 20 MIN/ 0.5 ML ALIQUOT (AS MALTOSE)
GA	H_2O	6.59
H_2O	H_2O	0.55
H_2O	GA	0.72

* Amount of GA added to endosperm solution was 200 μg/3 ml solution.

** Amount of GA added to assay solution was 100 μg, or about three times amount of GA that theoretically might have been carried over from solution of GA-treated endosperm.

Aliquots of the combined solutions were used as the enzyme source in the subsequent amylase assay. The results are presented in table III, as mg maltose produced in 20 minutes by a 0.5 ml enzyme aliquot.

The presence of GA in the assay induces almost no increase in amylase activity. On the other hand, the presence of GA during the endosperm incubation resulted in a large amount of amylase activity in the solution surrounding the endosperm. The results enable us to rule out the first possibility presented above, and substantiate the remaining hypothesis, i.e., that the observed maltose release is probably due to the activity of amylase and that GA induces an increase in the number of active amylase molecules derived from the endosperm itself, in the complete absence of the embryo.

GENERAL CONCLUSIONS

There is no longer any doubt that gibberellins are present in plants and that their action is hormonal in nature, (i.e., produced in a plant, and in low concentrations regulating physiological processes usually at a site some distance from the one of production). However, although many of the effects of gibberellins so far described are undoubtedly associated with their natural actions in the plant, many are also due to the high concentrations applied. In low, or what have been termed physiological concentrations the gibberellins have been shown to influence the genetic control of dwarfing, elicit typical long-day responses, replace vernalization requirements, etc. (3), and, from the results presented, it now appears possible to duplicate, at least in terms of starch hydrolysis, the effect of the embryo on the endosperm by treatment of the endosperm with GA.

As the concentration of GA applied to the endosperm increases, liberation of reducing sugars also increases. The effect is due to enzymatic activity and the enzyme involved has been shown to be sensitive to changes in buffer pH and concentration. The effects of these conditions on sugar release are similar to their effects on amylase activity. It is obvious that the effect of GA is not limited to the embryo as postulated by Pollack (19). That there is a fairly large amount of β-amylase present in the endosperm is in good agreement with the work of Engel (5) and rules out the necessity of postulating secretion by the embryo as a source of β-amylase activity in the endosperm. The results further substantiate Hayashi's (7) report that GA increases the amount of enzyme present and not the activity of the enzyme.

Lockart (9) has demonstrated that young, meristematic tissue is probably the site of production of a GA-like hormone, and although such a substance has not yet been identified in the embryo of germinating seeds, that tissue fulfills the conditions so far elaborated for an active productive site. It is tempting to postulate that an active production by the embryo of a GA-like substance occurs during germination and that an effect similar to that described, occurs in vivo. In such a situation, the GA-like substance might diffuse or be transported from its production site to a point of action (the endosperm in the case of barley) where it stimulates enzymic activity (amylase activity in the barley endosperm). Unfortunately, these experiments do not demonstrate a direct relationship between sugar production and the appearance of amylase activity in vivo. As a consequence, we can only infer that if amylase activity is indeed important in the endogenous hydrolysis of starch, then the GA-like substance may fulfill an hormonal role in the execution of embryonic control over a process in the endosperm which is essential for continued growth of the seedling.

The specificity of the reaction, with regard to the effectiveness of compounds other than GA on sugar liberation, has not been determined, and consequently, the reaction is not recommended as a test for the presence of GA. As we have seen (table II) other organic acids and several salts have no ability to induce sugar release, however, and after a more extensive examination, which is in progress, the reaction may well prove to be a reliable bioassay for GA.

Stimulation of the respiration of intact seeds by treatment with GA has recently been reported (15), and the measure of CO_2 produced was proposed as a quantitative assay for gibberellins. Although CO_2 evolution may indeed have quantitative significance, note that the seeds were not sterilized, and it has been found (unpublished information) that GA increases the liberation of reducing sugars into the surrounding medium by intact seeds as well as endosperm.

After completion of this paper, an article by M. Radley (20) appeared confirming the supposition that gibberellins do occur endogenously in barley and that they apparently increase in amount during germination.

SUMMARY

The effect of GA on the liberation of reducing sugars and amylase from the barley endosperm was

investigated. Concentrations from 2×10^{-4} μg to 2×10^{2} μg GA per 3 ml solution were active in producing increasing amounts of both maltose and glucose from the endosperm. At pH 5.5, 0.01 M acetate and citrate buffers inhibit the GA-induced reducing sugar release, while at a concentration of 0.001 M phthalate and acetate buffers show inhibitory effects, although acetate inhibition is much reduced from what it was at the higher concentration. The production of reducing sugars from starch in an amylase assay, was greatly increased when endosperm were pretreated with GA, providing evidence for the conclusion that GA increases the water soluble, amylolytic activity of the endosperm.

Acknowledgement

The author wishes to express his appreciation for the technical assistance of Miss Jan Sincock.

Literature Cited

1. Ballou, G. and J. Luck 1941. Effects of different buffers on the activity of β-amylase. Jour. Biol. Chem. 139: 233–240.
2. Barnell, H. R. 1937. Analytical studies in plant respiration VII. Aerobic respiration in barley seedlings and its relation to growth and carbohydrate supply. Proc. Roy. Soc. (London) B. 123: 321–342.
3. Brian, P. W. 1959. Effects of gibberellins on plant growth and development. Biol. Rev. 34: 37–84.
4. Brian, P. W. and H. G. Hemming 1955. The effect of gibberellic acid on shoot growth of pea seedlings. Physiol. Plantarum 8: 669–681.
5. Engel, C. 1947. The distribution of the enzymes in resting cereals. I. The distribution of the saccharogenic amylase in wheat, rye, and barley. Biochim. Biophys. Acta 1: 42–49.
6. Hall, R., G. Harris, and I. MacWilliam 1956. Carbohydrates in malting and brewing. V. Further studies on the carbohydrates of barley, malt, and wort. Jour. Inst. Brewing 62: 232–238.
7. Hayashi, T. 1940. Biochemical studies on "Bakanae" fungus of rice. VI. Effect of gibberellin on the activity of amylase in germinated cereal grains. Bull. Agr. Chem. Soc. Japan. 16: 531–538.
8. Kirsop, B. and J. Pollack. 1958. Studies in barley and malt. XII. Interaction between the embryo and the endosperm of barley during malting. Jour. Inst. Brewing 64: 227–233.
9. Lockhart, J. 1957. Studies on the organ of production of the natural gibberellin factor in higher plants. Plant Physiol. 32: 204–207.
10. Merry, J. and D. Goddard. 1941. A respiratory study of barley grain and seedlings. Proc. Rochester Acad. Sci. 8: 28–44.
11. Morris, E. 1958. Effect of gibberellic acid upon the germination of barley. Chemistry and Industry P. 97.
12. Munekata, H. and S. Kato. 1957. Studies on "Bakanae" fungus. XXXX. Application of gibberellin to malting industry. Bull. Brewing Soc. Japan 3: 1–10.
13. Myrback, K. and G. Neumuller. 1950. Amylases and the hydrolysis of starch and glycogen. In: The Enzymes, Sumner and Myrback, eds. Vol. 1 Pp. 653–727. Academic Press, New York
14. Nelson, N. 1944. A photometric adaption of the Somogyi method for the determination of glucose. Jour. Biol. Chem. 153: 375–380.
15. Nielsen, N. and G. Bergqvist. 1958. The stimulation of the respiration of seeds with gibberellic acid and its analytical significance. Physiol. Plantarum 11: 329–331.
16. Osborne, T. B. 1924. The Vegetable Proteins. 2nd ed. Longmans, Green and Co., London
17. Paleg, L. G. 1959. Citric acid interference in the estimation of reducing sugars with alkaline copper reagents. Anal. Chem. 31: 1902–1904.
18. Paleg, L. G. and D. Aspinall. 1958. Inhibition of the development of the barley spike by gibberellic acid. Nature 181: 1743–1744.
19. Pollack, J. 1958. Growth substances in relation to dormancy in barley. Chemistry and Industry. Pp. 387–388.
20. Radley, M. 1959. Occurrence of gibberellin-like substances in barley and malt. Chemistry and Industry Pp. 877–878.
21. Sandegren, E. 1949. Barley proteins in brewing. Food (London). 18: 292.
22. Schmidhauser, T. 1955. Die Verteilung der loslichen Zucker im keimenden Gerstenkorn. Ber. schweiz. bot. Ges. 65: 302–342.
23. Somogyi, M. 1952. Notes on sugar determination, Jour. Biol. Chem. 195: 19–23.
24. Stanley, R. 1957. Krebs cycle activity of mitochondria from endosperm of sugar pine seed (*Pinus lambertiana* Dougl.). Plant Physiol. 32: 409–412.
25. Whelan, W. 1958. Starch and similar polysaccharides. In: Encyclopedia of Plant Physiology, W. Ruhland, ed. Vol. 6. Pp. 154–240. Springer-Verlag, Berlin

COHESION OF WATER IN PLANTS [1, 2]

W. E. LOOMIS, R. SANTAMARIA-P., AND R. S. GAGE [3]

DEPARTMENT OF BOTANY AND PLANT PATHOLOGY, IOWA STATE UNIVERSITY, AMES

Forces applied through the cohesion of water have been generally accepted as the cause of the rise of the transpiration stream since the early publications of Dixon and Joly (8). The water absorbing forces of the leaf mesophyll cells can be shown to be adequate to lift water to the height of the leaves. The cohesion theory assumes that the diffusion of water from the non-collapsible xylem elements in contact with these leaf cells creates a state of tension within interconnected water columns in the xylem. This tension is possible because of the cohesion of water molecules and their adhesion to the hydrophilic walls of the xylem elements. Tension in the water columns is assumed to lift water from the roots to the leaves and, in addition, to reduce the free energy of the water in the root xylem until water diffuses from the soil into the root during passive absorption of water.

Greenidge (9) and Hildebrand (13) have discussed briefly the various methods of estimating the internal pressure and potential cohesion of water. These give values varying from about 4,000 to nearly 40,000 atmospheres. They are estimates, however, rather than measurements and it is not established that cohesion is equivalent to internal pressure.

The first successful attempt to measure cohesion in water experimentally seems to have been made by Berthelot (2) in 1850. He obtained values to 50 atmospheres by a method similar to that used later by Dixon and Joly (8). The results obtained by the Dublin workers were summarized by Dixon (7) in 1914. Estimates of cohesion by distortion of glass tubes were about eight atmospheres while those based on the Berthelot method were as high as 200 atmospheres. Ursprung (26) calculated that tensions on the order of 300 atmospheres were reached in the annulus cells of discharging fern sporangia, and King (15) concluded from an analysis of the dynamics of the fern annulus that cohesion values greater than 10 and less than 10,000 atmospheres were concerned. Direct measurements of cohesion (1, 24), in contrast, give maximum values of only 2 or 3 atmospheres, enough to demonstrate the reality of the process, but not to lift the transpiration stream in tall trees.

A number of recent workers have raised further questions concerning the function of the cohesion of water in plants; much of this work is discussed by Greenidge (12). Scholander et al (20) were unable to fit hydrostatic pressures in transpiring grape vines into a cohesion pattern. Measured pressures did not indicate cohesive tension at any time, and hydrostatic pressures in transpiring tall vines were higher at the top rather than lower, as they should have been if the transpiration stream was under tension. Their data recall the conclusion of Copeland (6) that the water columns in plants receive important support from unknown sources. In a study of moisture relations in tall lianas Scholander et al (21) found that allowing the vessels of a cut vine to become plugged with air caused a lowered hydrostatic pressure in the plant but did not reduce the rate of water uptake, indicating that water movement was shifted to the numerous tracheids of the stem. Again they found no direct evidence of cohesive tensions.

Greenidge (11) has performed numerous experiments in which a dye was injected into the xylem of trees after severing all vessels of the stem by two or more opposing saw cuts. Dye not only moved readily to the top of such trees, but it completely stained the wood immediately below and above the saw cuts, indicating capillary movement under low tension, and showing no evidence of the rupture of stressed water columns. In other experiments the dye moved to the top of tree trunks from which all leafy branches had been removed and all vessels severed at one or more points.

In spite of the difficulties of demonstrating appreciable values of tension in the water columns of plants, plant physiologists continue to assume that high tension values are readily obtainable. Bonner (3) has suggested that water can move in the walls of air filled vessels, with cohesion providing the large forces required for such movement. And Went (27) has suggested that negative turgor pressures, or tensions, on the order of 400 atmospheres are responsible for the low rates of transpiration observed in some desert plants.

The experiments of Dixon and Joly (8) and of Ursprung (26) are the major basis for assuming the possibility of high water tensions in plants. Because of the importance of their work, and because of irregularities in the methods and data, we have repeated these classical experiments.

EXPERIMENTS WITH FERN ANNULUS

Ursprung's experiments (26) were repeated with sporangia from a tropical species of Polypodium. Fresh sori were collected in the greenhouse and kept moist until used. A number of sporangia were then suspended in cotton wool nets and sealed over a series

[1] Received revised manuscript July 14, 1959.

[2] Journal Paper No. J-3593 of the Iowa Agricultural and Home Economics Experiment Station, Ames. Project No. 1139.

[3] Present address: Dept. of Physics, Ontario Agricultural College, Guelph, Ontario, Canada.

of sulfuric acid solutions in large Van Tieghem cells. The cultures were observed at frequent intervals with a low power microscope and the percentage of discharged sporangia estimated.

When held at 20° C only the full and half strength acid caused any of the sporangia to open in 30 minutes (table I). According to Ursprung's procedure, sulfuric acid with a sp. gr. of 1.27 would have been considered to have a vapor pressure equivalent to that of the annular cells. The vapor pressure at the surface of the cells of the annulus would therefore be assumed to have been 11.4 mm, equivalent to a relative humidity of 65 %, and the entire reduction in the vapor pressure from that of pure water would have been assigned to cohesion through a calculation of surface tension effects. These calculations are based upon data obtained by Thompson (23) in capillary tubes and upon the usual capillary equation. These equations were used by Ursprung without regard for the very considerable adsorption effects that are undoubtedly present.

TABLE I

PERCENTAGES OF FERN SPORANGIA OPENED OVER SULFURIC ACID SOLUTIONS

SP. GR. OF ACID	TIME IN HOURS					
	0.5	1.0	2.0	4.0	6.0	24.0
1.83	10	10	30	50	90	100
1.51	10	10	25	50	70	100
1.27	0	4	25	40	70	100
1.16	0	2	15	30	40	100
1.105	0	0	0	0	5	20
1.09	0	0	0	0	0	0
1.08	0	0	0	0	0	0

Applying Ursprung's calculations to the data of table I, we obtain an assumed value for water tension and cohesion of nearly 500 atmospheres. Thirty minutes was far too short a time to obtain equilibrium between the annulus cells and the acid solutions (table I). After 24 hours, 20 % of the sporangia had opened over acid with a sp. gr. of 1.105, and after 3 days all of these sporangia were discharged and none had opened above acid with a sp. gr. of 1.09. The indicated vapor pressure was now 16.25 mm and the relative humidity at equilibrium 93.5 instead of 65 %. Even these results indicate a cohesion value of about 80 atmospheres by Ursprung's method of calculation.

The method, however, is based upon at least five questionable assumptions: I. that there are well defined pores reaching the surface of the annulus cells, II. that no forces affect the vapor pressure within these pores other than the capillary tension of water, III. that the capillary equation is applicable at pore diameters where adsorption forces are undoubtedly present and probably large, IV. that no solutes affect the osmotic pressure of the cell contents or the surface tension of water within the pores, and V. that there are no membrane effects on the movement of water.

Mueller et al (17) and Schieferstein and Loomis (19) have shown that the pores of the cellulose wall do not reach the surface of exposed cells, but are covered by pectins or hemicelluloses with a very much finer structure than cellulose, and that these substances in turn are covered by the primary cuticle. The 24 hour reading in table I indicates a surface pore diameter of more than 300Å. No external cell surface of hundreds examined in this laboratory has shown any evidence of surface pores at magnifications up to 50,000 diameters, where pores of 50Å or less should have been visible. The question of adsorption forces within small pores has not been satisfactorily resolved. Thompson (23) thought that adsorption would invalidate his equation at pore diameters of 5,000Å, 50 to 100 times larger than the values used by Ursprung. The same difficulty applies to the use of the capillarity equation. Osmotic and surface effects within the cells and wall would undoubtedly be significant and the latter probably large.

Evaporation across plant or artificial membranes was shown by Boon-Long (4) to be considerably slower than would be expected on the basis of the vapor pressure of water in the solution. Thut (25) also has shown that vapor pressures within transpiring leaves may be very much lower than are commonly assumed on the basis of the osmotic pressures of the leaf sap. He found relative humidities within the intercellular spaces of wilted leaves as low as 55 %, when values near 99 % were obtained with the leaf tissues. If Ursprung's calculations had been applied to these data they would have indicated a cohesive tension of well over 600 atmospheres in the leaf mesophyll cells. Since cohesive forces cannot be developed in cells with nonrigid walls, this calculation would be obviously invalid.

If the opening and discharge of the fern annulus requires water tensions of the magnitude calculated by Ursprung, these tensions would build up to a maximum during the recurving of the annulus structure. Evidence that no important build-up occurs is available from the observation that all sporangia that showed rupturing of the lip cells were eventually discharged. Thus all sporangia held over acid with a sp. gr. of 1.105 completed the cycle and discharged when left for 3 days, but *none opened* over acid at the next lower concentration. Moreover, discharged sporangia can be soaked in water and the cycle repeated with all mechanical resistance to opening removed. Such recycling follows the same path as the original opening and discharge.

These results indicate that a general, membrane or solute effect rather than cohesive tension is the major factor effective in reducing evaporation from the annulus. It is postulated that partial drying of the annulus cells causes the water surface to be withdrawn into the cellulose-pectin wall, where adsorption forces are the primary cause of the large reduction of vapor pressure which is obtained. The same explanation would apply to the observations of Thut

(25) and Went (27). The snap closing of the annulus is evidence of cavitation in the water of the annular cells; some cohesive tension undoubtedly is built up in these cells. If we accept King's (15) calculations, a minimum tension of 10 atmospheres should be present. Since, however, he was able to conclude only that a value between 10 and 10,000 atmospheres should be required, his calculations are not useful for quantitative purposes.

The fern annulus experiments may be considered to demonstrate the presence of cohesion. They offer no support for the high tension values estimated by Ursprung, and there is no present basis for concluding that they have or can yield quantitative determinations of the cohesion of water.

Experiments with Sealed Glass Tubes

A large number of estimations of the cohesion of water have been made with the Berthelot method as modified by Dixon and Joly (8). Pyrex glass tubes of varying size and about 20 centimeters long were carefully cleaned with hot NaOH and chromic acid solutions, sealed at one end and drawn to a neck at the other. The tubes were annealed by heating for 0.5 hours at 550° C and cooling in 2 hours or more to remove stresses and insure uniform physical reactions in the glass. They were then filled with water or solution, the volume of the contained liquid adjusted at standard temperatures, and the neck of the tubes sealed with a small flame and a reduced pressure. Most of the tubes were filled with well boiled liquid and handled rapidly to reduce adsorption of gases by the liquid. Some were filled with unboiled water and sealed at the relatively high pressure of 700 mm Hg. Others were filled with thoroughly boiled, glass-distilled water, cooled rapidly and sealed at a pressure of 1 to 2 mm to obtain a minimum of dissolved gases.

Determinations consisted of heating one of these tubes slowly in a water bath with constant stirring. Heat was supplied by microburners or controlled electric heaters. Water expands more rapidly with heating than glass, so that the free space in the tip of the tubes closes as the temperature is raised. Final heating was at the rate of 1° C in 5 minutes to insure uniform temperatures in the bath and tube. Closing was observed with a microscope; the temperature of the bath at closing was recorded as t_o. The bath was then allowed to cool slowly with stirring until the bubble re-formed, usually with a sharp click, indicating tension and cohesion within the tube. The breaking temperature was recorded as t_1, and the indicated cohesion calculated with a modification of Dixon's (7) equation:

$$T = \frac{(a-g)\ (t_o-t_1)}{B[1-a(t_o-t_1)] + \frac{1-g(t_o-t_1)}{R^2 - r^2} \times \left(\frac{r^2}{k} + \frac{R^2}{n}\right)}$$

where T = tension and cohesion in atmospheres, a is the temperature coefficient for the expansion of water, g the coefficient for Pyrex glass, B the coefficient of compressibility of water at t_1, R and r the external and internal radii of the tube, k the volume elasticity and n the modulus of rigidity of the glass. Values for the constants were obtained from the Landolt-Börnstein tables, or from the Corning Glass Co., Corning, N. Y. Values of B for various temperatures were plotted and the figures used were taken from the smoothed curves. An approximate value of T could be obtained from $T = 10(t_o-t_1)$.

Dixon (7) obtained significantly higher values of T with his thinner walled tubes, suggesting an error in correcting for the characteristics of the glass; we accordingly tested tubes of varying size and wall thickness. The results are shown in table II. Our highest values were obtained with the thick-walled tubes, W-I and W-III; note, however, that the third highest value was obtained with the thin-walled W-VII, and that calculated cohesion values were not correlated with tube size or wall thickness.

Two other results show in the table, the high variability among replicate determinations with the same tube, and the generally lower values obtained with the tubes of the S series which contained a detergent, Alconox, that reduced the surface tension of the water to half its normal value. Surface tension

Table II

Tube Characteristics and Calculated Tension Values

Tube	External radius, R (cm)	Internal radius, r (cm)	Wall thickness (cm)	No. of determinations	Calculated T values (atm.)	
					Range	Average
W-I	0.500	0.250	0.125	4	103-228	171.7
W-II	0.595	0.415	0.090	3	73-101	83.1
W-III	0.495	0.260	0.117	2	129-175	152.0
W-IV	0.490	0.260	0.115	10	44-103	74.4
W-V	0.490	0.260	0.115	4	29-107	70.7
W-VI	0.299	0.196	0.051	5	56-142	102.1
W-VII	0.299	0.196	0.051	11	106-152	134.3
W-VIII	0.299	0.196	0.051	11	42-92	62.3
S-I	0.299	0.196	0.051	8	28-61	43.5
S-II	0.299	0.196	0.051	5	24-59	35.5
S-III	0.299	0.196	0.051	3	78-128	106.2
S-IV	0.299	0.196	0.051	5	39-52	51.6

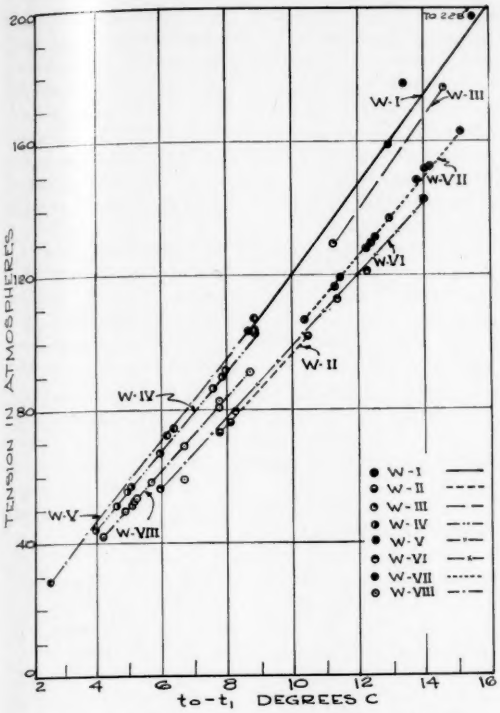


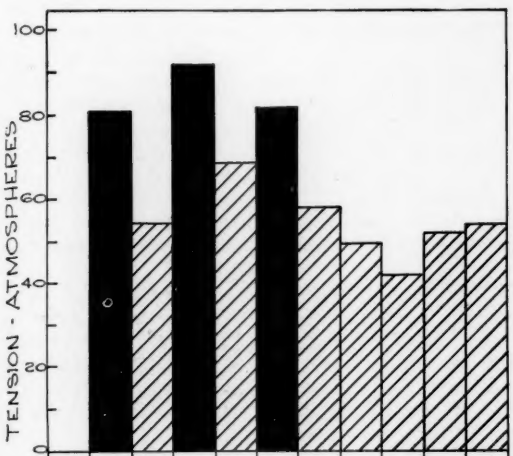
FIG. 1. Variability of replicate cohesion readings on individual tubes. The vertical spread arises from the variation of B with different temperatures of t_I, even though t_o-t_I was the same.

is an expression of cohesive forces; measurements of cohesion are dependent upon surface tension around gas nuclei within the liquid and upon interfacial tensions between liquid and glass. The average value of cohesion for the water-filled tubes in table II was 98.8 atmospheres, while that with the detergent solution was 52.5 atmospheres. The difference was significant far beyond the 1 % level in spite of the high average value for tube S-III. The direction and magnitude of the effect agrees with expectation, and is evidence that cohesive tensions are developed in the tubes.

The differences in the average values obtained with the different tubes were large, more than 100 atmospheres among tubes of the W series, and highly significant statistically. Also, repeat readings on individual tubes varied widely (fig 1), with differences of 50 atmospheres common and a maximum spread of 125 atmospheres. A similar result was obtained by Dixon (7), it might be assumed that differences in gas nuclei or other factors affecting the cavitation of water (14, 18) were responsible for the variability. When it was found, however, that a second reading made as quickly as possible after a first, always gave a lower value for apparent cohesion than one made after the tubes had stood for some time, a further study was undertaken.

Apparent cohesion values obtained with a single tube are shown in figure 2. The solid bars represent values obtained after the tube had stood overnight, the cross-hatched bars, repeat tests. Of the possible explanations for these results, plastic deformation of the glass tube was discarded as being too improbable. Glass is almost perfectly elastic at the temperatures used here. The persistence of cavitation nuclei as a causal factor was eliminated by the observation that the variation was primarily in the closing temperature, t_0. The closing temperature would have been expected to be a function of the volume of the glass and the liquid, and hence constant, while a variable cavitation would have shown in the breaking temperatures. Records for three tubes in the W series on which the largest number of readings were available are shown in table III. Variations in values of the closing temperature (t_0) were more than three times those in the breaking temperature (t_1); the calculated cohesion values were highly correlated with closing temperatures alone.

In his original publication in 1850 Berthelot (2) noted that temperatures far above the minimum closing value were sometimes needed to force the gas bubble present in the cooled tube into solution in the expanding liquid. In one of his experiments a temperature of 75° C was required to obtain gas solution in a tube which should have closed at about 30° C. Temperley and Chambers (22) had noted the same difficulty, and had resorted to direct measurements of liquid volume changes, rather than to temperature changes, as indicators of cohesive forces. Prelimi-



t_o	72.3	70.0	73.5	71.5	72.5	70.2	69.5	68.8	69.1	69.7
t_1	64.5	64.6	64.8	64.8	64.7	64.5	64.6	64.6	64.0	64.4

FIG. 2. Calculated cohesion values after a tube had stood overnight (solid graphs) and immediately after a previous test (barred graphs).

nary experiments in our work showed that closing occurred at lower temperatures if the tube was removed from the bath and inverted at intervals to speed solution of the gas bubble. In one test the closing temperature was lowered 7° C by this treatment. Also, higher closing temperatures were obtained when the bubble was left in the pointed end of the tube rather than in the rounded end that was closed before filling, and in which a broader contact between bubble and liquid was obtained.

To check the problem further, three series of tubes were prepared. U-700 tubes were filled with unboiled water and closed at a pressure of 700 mm Hg to obtain a maximum quantity of dissolved gases; B-700 tubes were filled with carefully boiled water, handled rapidly and closed at 700 mm Hg; B-2 tubes were prepared to give the minimum content of dissolved gases. An extra neck was drawn on the tubes and they were sealed dry under a high vacuum. The tubes were then immersed in thoroughly boiled water that had been cooled in an atmosphere of water vapor, and the seal broken to fill the tubes. These were then cooled rapidly in an ice bath and resealed at a pressure of 1 to 2 mm Hg, below the vapor pressure of ice. The absence of cavitation at these low pressures is evidence that large gas or similar cavitation nuclei had been eliminated.

Data obtained with five tubes representing the three methods of filling are shown in table IV. A sixth tube filled with unboiled water apparently contained a cavitation nucleus and gave consistently low values for cohesion. The heating procedure was changed for these determinations. Instead of heating slowly until the bubble disappeared, heating was stopped when the bubble reached a small size, 5 units on the microscope scale used. The temperature at this point was recorded as t_h, or the high temperature. The bath and tube were then cooled slowly until the bubble started to enlarge. The temperature drop over which the bubble size remained apparently unchanged was as much as 7.7° C, suggesting that pressures on the order of 75 atmospheres had been built up in the tubes. The temperature when the bubble in the cooling tube started to enlarge was recorded as t_g, the bubble growth temperature. The bath was then alternately heated and cooled over a narrow range for as long as an hour to permit gases to dissolve in the water. At the end of this period the tubes closed at a temperature (t_o) *which was in all readings only* 0.1° C *above* t_g.

The second part of table IV shows redeterminations made on the individual tubes immediately after completing the first determination. Even though the tube had been filled and any gases present dissolved a few minutes before, the high temperature still averaged 4° C above the closing temperature. It is significant that all of the rerun effect was in this overshoot temperature, and that closing temperatures within each of the tubes were identical in the two runs within the error of reading. A third run gave results identical with the second. Although not shown in the table, it was included in the calculated average values for cohesion.

The experiments reported in table IV were intended to demonstrate the effects, noted by Berthelot (*2*), of the slow solubility of gases on closing temperatures, and hence on estimated cohesion. The results are inconclusive. The slow heating method eliminated the large variations previously obtained within and between tubes. Instead of a range of 29 to 228 atmospheres, as shown in table II, the range was now 24 to 31, or with tube B-2′ omitted, 24 to 25 atmospheres. There was, however, no consistent effect of the varying quantities of gas within the tubes. Some dissolved gases may remain in water after prolonged boiling, but there should have been enough difference in the B-2 and in the B-700 and U-700 tubes to have shown in the t_h temperatures. Closing temperatures, t_o, were attained after allowing any necessary time for the solution of gases from the

TABLE III

CORRELATIONS BETWEEN CLOSING TEMPERATURES (t_o = X) AND COHESION (T = Y)

READING	TUBE W-IV			TUBE W-VII			TUBE W-VIII		
	t_o(=X)	t_l	T(=Y)	t_o(=X)	t_l	T(=Y)	t_o(=X)	t_l	T(=Y)
1	76.8	67.9	102.0	73.5	59.3	152	72.3	64.5	80.9
2	76.0	68.0	91.0	70.3	59.9	106	70.0	64.6	54.8
3	72.0	68.0	43.7	70.2	58.9	116	73.5	64.8	91.8
4	73.5	67.5	66.6	71.2	58.8	129	71.5	64.8	69.3
5	74.0	68.9	56.9	71.3	59.0	128	72.5	64.7	81.3
6	75.5	67.6	89.4	70.9	59.4	119	70.2	64.5	58.2
7	77.1	68.2	103.0	71.2	58.7	130	69.5	64.6	49.5
8	72.8	67.8	55.2	71.3	58.3	136	68.8	64.6	42.0
9	72.5	67.9	50.6	73.1	59.2	148	69.1	64.0	51.4
10	75.7	68.1	86.1	73.5	58.3	162	69.7	64.4	53.9
11	...	...	...	73.6	59.5	151	69.3	64.1	52.5
Avg.	74.6	68.0	74.4	71.8	59.0	134.3	70.6	64.5	62.3
		r = 0.98			r = 0.96			r = 0.99	

TABLE IV

TEMPERATURES AND COHESION VALUES IN BERTHELOT TUBES FILLED WITH VARYING GAS PRESSURES AND HEATED TO ALLOW SOLUTION OF GASES BEFORE CLOSING

READING	TUBES					
	B–2	B–2′	B–700	B–700′	U–700	AVG.
A. Determination with tubes after standing						
T_h (high)	65.5	64.0	66.5	63.0	66.0	65.00
T_g (holding)	61.4	56.8	58.8	57.8	59.8	58.92
T_h-T_g (overshot)	4.1	7.2	7.7	5.2	6.2	6.08
T_o (closing)	61.5	56.9	58.9	57.9	59.9	59.02
T_l (breaking)	58.9	53.9	56.6	55.8	57.6	56.56
T_o-T_l (cohesion)	2.6	3.0	2.3	2.1	2.3	2.46
B. Second determination immediately after A						
T_h (high)	63.5	61.2	64.2	63.0	64.0	63.18
T_g (holding)	61.4	56.9	58.9	57.7	59.8	58.94
T_h-T_g (overshot)	2.1	4.3	5.3	5.3	4.2	4.24
T_o (closing)	61.5	57.0	59.0	57.8	59.9	59.04
T_l (breaking)	59.0	53.9	56.5	55.3	57.6	56.46
T_o-T_l (cohesion)	2.5	3.1	2.5	2.5	2.3	2.58
Avg. cohesion in atm.	25	31	25	24	24	25.8

bubble. The temperature, t_h, however, was reached by continuous heating at uniform rates. The overshoot temperature, t_h-t_g, should, therefore, have shown differences proportional to the gas content of the tubes if only gas solution was involved. Such differences were not shown consistently. These results may indicate that some structural discontinuity of water is present here, in addition to the gas-solution effect.

One clear-cut result is shown by the data of table IV. The high values for cohesion reported by Dixon (7) and estimated in table II are not valid. The average cohesion estimated in table II is 98.8 atmospheres, while that for table IV is only a fourth as much, or 25.8 atmospheres. Temperley and Chambers (22) calculated cohesion from changes in the volume of water at cavitation and obtained an average value of 32 atmospheres. Meyer (16) measured cohesion from the distortion of a coiled glass tube at the instant of cavitation and found an average value of 26.8 atmospheres. Three distinct methods thus give agreement on a value of 25 to 30 atmospheres for the cohesion of water in glass. Only the centrifugal method of Briggs (5) is left with high values, and this agrees with the others when run at temperatures near freezing.

Since cavitation in the tubes can be observed to occur within the body of the liquid or, alternatively, at the water glass boundary without altering the estimates of cohesion, the values obtained are considered to be characteristic of water and to be independent of the enclosing material. We expect, therefore, that the maximum realizable values of cohesion in plants will be comparable; Greenidge (10) has, in fact, estimated the maximum fracturing tension of water columns in a maple tree at 26 atmospheres. These values are, of course, adequate to lift water in the tallest trees, provided cohesion in the xylem is not less than in glass and resistance to water movement is not excessive. They do not support theories of water transport which require dependable, high values for the cohesion of water.

SUMMARY

The assumption that cohesion values in water are more than adequate to explain the rise of the transpiration stream in the tallest plants has been based upon the classical experiments of Ursprung (26) and Dixon (7). It is pointed out in our discussion that Ursprung's calculations of cohesion in the fern annulus

are based upon a number of unproven and improbable assumptions. The method is considered unsuitable for quantitative estimates of cohesion.

Dixon's experiments have been repeated and a maximum value of 25 to 30 atmospheres for the cohesion of water obtained, rather than the 200 atmospheres claimed by Dixon. This value agrees with those obtained by two related but independent methods (16, 22), and is considered the best estimate of the cohesive strength of water presently available. A cohesion of 25 to 30 atmospheres is, of course, adequate to lift water to the top of the tallest trees. It does not, however, permit the development of theories of water transport entailing high resistance factors.

Literature Cited

1. Askenasy, E. 1897. Beiträge zur Erklärung des Saftsteigens. Verh. Naturhist.-Med. Ver. Heidelberg 5: 429–448.
2. Berthelot, M. 1850. Sur quelques phénomènes de dilatation forcée des liquides. Ann. Physik. Chemie. Ser. 3. 30: 232–237.
3. Bonner, J. 1959. Water transport. Science 129: 447–450.
4. Boon-Long, T. B. 1941. Transpiration as influenced by osmotic concentration and cell permeability. Amer. Jour. Bot. 28: 333–343.
5. Briggs, L. J. 1950. Limiting negative pressure of water. Jour. Appl. Phys. 21: 721–722.
6. Copeland, E. B. 1902. The rise of the transpiration stream: an historical and critical discussion. Bot. Gaz. 34: 161–193; 260–283.
7. Dixon, H. H. 1914. Transpiration and the Ascent of Sap in Plants. Macmillan, London.
8. Dixon, H. H. and J. Joly. 1894. On the Ascent of Sap. Proc. Roy. Soc. (London) 57: 3–5.
9. Greenidge, K. N. H. 1954. Studies in the physiology of forest trees. I. Physical factors affecting the movement of moisture. Amer. Jour. Bot. 41: 807–811.
10. Greenidge, K. N. H. 1955. II. Experimental studies of fracture of stretched water columns in transpiring trees. Amer. Jour. Bot. 42: 28–37.
11. Greenidge, K. N. H. 1955. III. The effect of drastic interruption of conducting tissue on moisture movement Amer. Jour. Bot. 42: 582–587.
12. Greenidge, K. N. H. 1957. Ascent of sap. Ann. Rev. Plant Physiol. 8: 237–256.
13. Hildebrand, J. H. 1916. Solubility. J. Amer. Chem. Soc. 38: 1452–1475.
14. Iyengar, K. S. and E. G. Richardson. 1958. Measurements on the air-nuclei in natural water which give rise to cavitation. British J. Appl. Phys. 9: 154–158.
15. King, A. L. 1944. The spore discharge mechanism of common ferns. Proc. Natl. Acad. Sci. U.S. 30: 155–161.
16. Meyer, J. 1911. Zur Kentniss des Negativen Drukes in Flüssigkeiten. Abhand. deut. Bunsen-Ges. 3: 1–53.
17. Mueller, L. E., P. H. Carr, and W. E. Loomis. 1954. The submicroscopic structure of plant surfaces. Amer. Jour. Bot. 41: 593–600.
18. Pease, D. C. and L. R. Blinks. 1947. Cavitation from solid surfaces in the absence of gas nuclei. Jour. Phys. & Colloid Chem. 51: 556–567.
19. Schieferstein, R. H. and W. E. Loomis 1956. Wax deposits on leaf surfaces. Plant Physiol. 31: 240–247.
20. Scholander, P. F., W. E. Love, and J. W. Kanwisher 1955. The rise of sap in tall grapevines. Plant Physiol. 30: 93–104.
21. Scholander, P. F., Berthe Rund, and Helge Leivestad 1957. The rise of sap in a tropical liana. Plant Physiol. 32: 1–6.
22. Temperley, H. N. V. and L. G. Chambers 1 The behaviour of water under hydrostatic tens I. Proc. Phys. Soc. (London) 58: 420–436.
23. Thompson, W. 1871. On the equilibrium of vap at the curved surface of liquids. Phil. Mag. 42: 448–452.
24. Thut, H. F. 1928. Demonstrations of the lifting power of evaporation. Ohio J. Sci. 28: 292–298.
25. Thut, H. F. 1939. The relative humidity gradient of stomatal transpiration. Amer. Jour. Bot. 26: 315–319.
26. Ursprung, A. 1915. Über die Kohäsion des Wassers im Farnannulus. Ber. deut. bot. ges. 33: 153–162.
27. Went, F. W. Presented at the 1959 Meetings of the Amer. Soc. Plant Physiologists. Stephen Hales Address.

LIGHT-DEPENDENT OXYGEN METABOLISM OF CHLOROPLAST PREPARATIONS. II. STIMULATION BY MANGANOUS IONS [1, 2]

HELEN M. HABERMANN [3]

DEPARTMENT OF BOTANY, UNIVERSITY OF MINNESOTA, MINNEAPOLIS

The importance of manganese for photosynthesis was first suggested by McHargue (17) and Bishop (2). Pirson (21) found that photosynthesis in manganese-starved Chlorella cells was inhibited despite the absence of visible chlorosis; this inhibition could be relieved by adding manganese to the medium. Emerson and Lewis (6) observed that manganese deficiency affected the maximum quantum yield of photosynthesis. Gerretsen (7) found that manganese deficiency caused a reduction in the rate of photosynthesis in oats. He surmised that manganese is specifically involved in the oxygen-liberating stage of photosynthesis and went on to study the effect of manganese salts on the potentials of electrodes immersed in chloroplast suspensions (8). Working with chloroplasts in the absence of Hill reagents, Gerretsen found that the electrode potential was changed markedly on addition of manganese and he suggested that peroxide is formed in the presence of manganese but not in its absence.

More recently Kessler (15, 16), from his studies of photosynthesis and photoreduction in manganese-deficient algae, provided convincing proof that the role of manganese in photosynthesis is mainly, if not exclusively, concerned with oxygen evolution.

Aside from the implication of manganese, we know very little about the process by which oxygen is evolved from the oxidized product of the splitting of water in photosynthesis. There is a comparable lack of experimental evidence concerning the role of manganese in photochemical oxygen production by isolated chloroplasts.

Mehler (18) was the first to show that oxygen could function as a Hill reagent, i.e., as an oxidant which is effective in promoting photochemical oxygen production by chloroplast preparations. In the Mehler reaction there is a light-dependent consumption of oxygen which is a consequence of the simultaneous reduction of oxygen by a photochemically generated reductant and evolution of oxygen from the oxidized product of photolysis. Added ethanol and excess catalase act as a "sink" for the peroxide produced by the reduction of molecular oxygen (13). The stoichiometry of the over-all reaction is such that two molecules of oxygen are consumed for every one produced (Mehler and Brown, 20).

More recently, Brown and Good (3) have shown that chloroplasts illuminated without addenda can produce and consume oxygen simultaneously. Both production and consumption are light dependent processes. The stoichiometry being 1 : 1, the chloroplasts thus effect an exchange of the oxygen of water for molecular oxygen dissolved in the suspending medium. In this exchange reaction (which differs from the Mehler reaction only in the method of removal of peroxide formed in the reduction of molecular oxygen), each mole of oxygen reduced by chloroplast preparations in the light is exactly balanced by the production of ½ mole from the oxidized product of photolysis and ½ mole from the decomposition of peroxide by the endogenous catalase of the chloroplasts. Because there is no net change of oxygen tension in the exchange reaction, it can be followed only by tracer oxygen techniques.

Mehler (19) tested the effect of added manganous ions on the Hill reaction and found no effect on the rate of oxygen evolution when quinone was used as the oxidant (whether or not catalase and ethanol were present). In the case of the Mehler reaction, however, he found that Mn^{++} increased the rate of net oxygen consumption. The manganese-stimulated rates were approximately the same as those which Mehler (19) had observed following quinone reduction. This stimulatory effect of manganese was catalytic; marked stimulation was given by 10^{-4} M Mn^{++} and the maximum effects were produced by a 10^{-3} M concentration.

Andreae (1) has reported a flavin-sensitized photooxidation of manganese ions and has suggested that the cyclic oxidation-reduction of manganese might explain the effects of manganous ions on oxygen uptake by illuminated chloroplast suspensions. Also, Kenten and Mann (14) have demonstrated a light-dependent oxidation of manganese in chloroplast preparations and have discussed its possible role in photosynthesis.

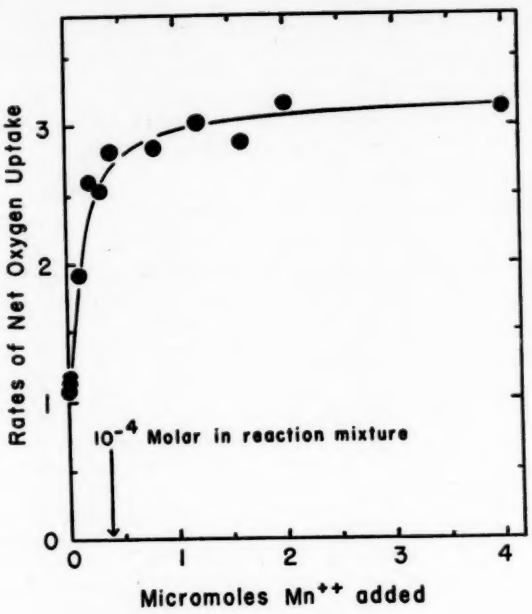
Mehler's observation of the manganese stimulation has been confirmed by Good and Hill (9) and extended to preparations from chard and Chenopodium.

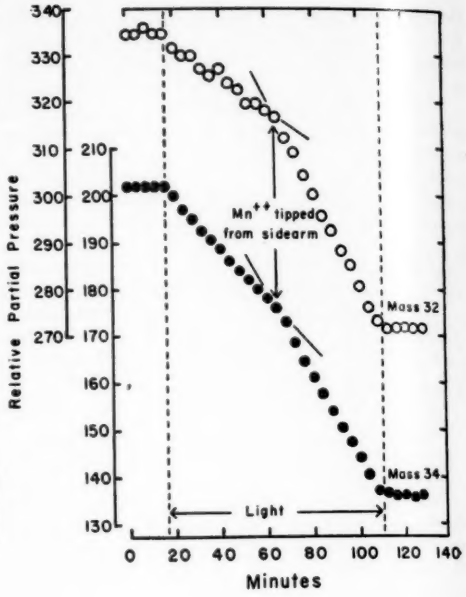
---

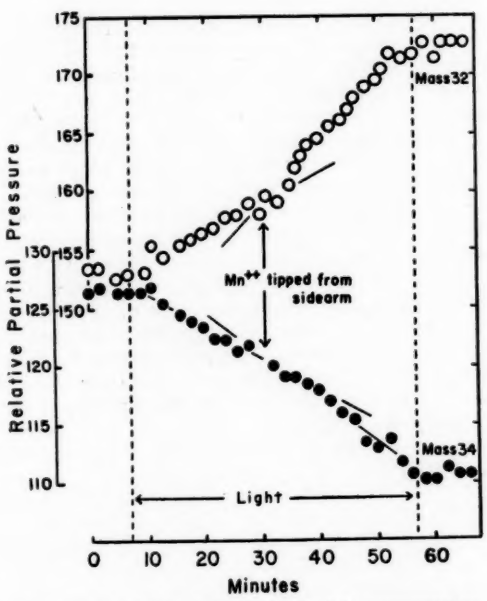
[1] Received July 16, 1959.

[2] The contents of this paper are part of a thesis submitted to the graduate faculty of the University of Minnesota in partial fulfillment of the requirement for the Ph.D. degree.

[3] Present address: Department of Biological Sciences, Goucher College, Towson, Baltimore 4, Md.







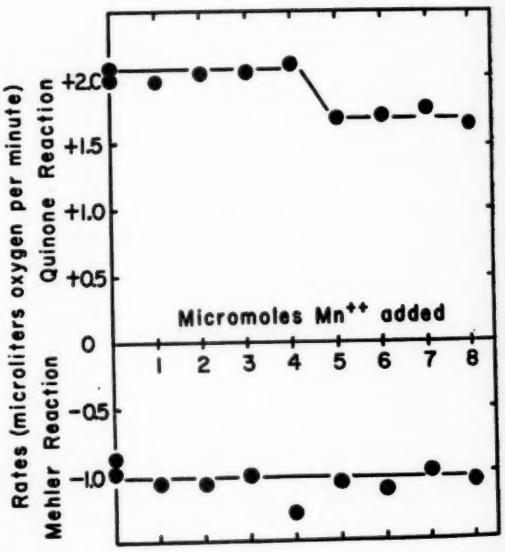


FIG. 1 (*top left*). Effect of Mn^{++} concentration on rates of net oxygen uptake in the Mehler reaction. Vessel contents: 3 cc chloroplast suspension (containing 0.5 mg chlorophyll), 6 mg catalase, 0.1 cc 50 % ethanol, 0 to 4 μM $MnCl_2$.

FIG. 2 (*top right*). Effect of adding Mn^{++} on time courses of normal and tracer oxygen in the Mehler reaction (Exp. 1). Vessel contents: 3 cc chloroplast suspension (containing 0.75 mg chlorophyll), 6 mg catalase, 0.1 cc 50 % ethanol, 6 μM $MnCl_2$ in sidearm. To convert partial pressure units of the ordinate to microliters oxygen multiply by 1.12.

They have suggested that increased oxygen uptake must have been due to hydrogen peroxide formation because it did not occur when the hydrogen peroxide-trapping catalase and ethanol were omitted.

This paper describes experiments in which tracer oxygen techniques were used to measure the effects of manganous ions on the rates of consumption and production of oxygen in the Mehler reaction and the related exchange reaction. The data indicated that manganese stimulation resulted from a reaction with the oxidized product of photolysis and there was indirect evidence of peroxide formation. The stimulation of light-dependent chloroplast reactions by manganous ions is compared with the stimulation by quinone and ascorbic acid previously described (Habermann, 10; Habermann and Brown, 11) and a scheme for the action of manganese is proposed.

Methods

Chloroplasts were prepared from mature leaves of *Phytolacca americana* L. according to procedures previously described (Habermann, 10). All reactions were run in conventional rectangular Warburg vessels. Light from a bank of fluorescent bulbs mounted vertically behind the water bath was reflected onto the bottoms of the experimental vessels by a mirror mounted at a 45° angle. Incident light intensity was approximately 400 ft-c. Manometric experiments were run at 15.6° C, isotope experiments at either 14.5° or 23° C. Adaptation of the mass spectrometer (CEC model 21–201) has been described by Brown, Nier, and Van Norman (4), and further modifications of the apparatus have been described by Johnston and Brown (12).

The gas phase for all manometric experiments was air. In experiments using tracer oxygen, the vessel containing 3 cc buffered chloroplast suspension plus addenda was attached to the leak assembly of the mass spectrometer and flushed with helium for 5 minutes. Oxygen enriched with mass 34 ($O^{16}O^{18}$) was then added so that the resulting gas phase was composed of 2 to 3 % oxygen in helium. The rates of oxygen consumption and evolution were calculated from time course records of the concentrations of mass 32 (ordinary oxygen) and mass 34 ($O^{16}O^{18}$) according to methods discussed by Brown and Weis (5).

Results

Effect of Mn^{++} Concentration: Figure 1 shows the rate dependence of the manganese-stimulated Mehler reaction on the amount of Mn^{++} added to the reaction mixture. Considerable stimulation of net oxygen uptake was observed at even the lowest concentration of added manganous ions and maximally stimulated rates were observed at concentrations above 10^{-4} M in the reaction mixture.

Tracer Oxygen Experiments: *I. Effect of Mn^{++} on the rates of oxygen consumption and production in the Mehler reaction*: A number of experiments were run in which $MnCl_2$ in solution was tipped from the sidearm of the experimental vessel after a period of unstimulated Mehler reaction in the light. The time courses of uptake of tracer and normal oxygen in a typical experiment are shown in figure 2. Following the addition of manganous ions to the reaction mixture, the rates of net consumption of both species were accelerated. The calculated rates of oxygen consumption and production in this experiment are given in table I (Exp. 1). Also in table I (Exp. 2) are data of a second representative experiment run with a different chloroplast preparation and at a higher temperature. In both experiments, the rates of uptake and production of oxygen in the Mehler reaction were accelerated following addition of Mn^{++} ions to the reaction mixtures. In this case, uptake was stimulated much more than production (the rate of oxygen uptake increased an average of 83 % after adding Mn^{++}, while the rate of oxygen production increased only 40 %).

II. Effect of Mn^{++} on the rates of uptake and production of oxygen in the exchange reaction: Experiment 3 (table I) was run with an aliquot of the chloroplast suspension used in experiment 1. Three cc of chloroplast suspension was pipetted into the main compartment of the vessel and $MnCl_2$ in solution was tipped from the sidearm after a period of unstimulated oxygen exchange in the light. The time courses of tracer and normal oxygen pressure changes in this experiment are shown in figure 3 and the calculated rates of oxygen consumption and production are summarized in table I (Exp. 3). In this case also, the rates of both uptake and production of oxygen were accelerated by the addition of manganous ions. In the exchange reaction, however, in contrast to the changes observed in the Mehler reaction, production of oxygen was stimulated more than uptake (the rate of uptake increased only 34 %, while the rate of production increased 61 %).

Effect on Rates of Quinone-Hill Reaction when Catalase and Ethanol are Present: The

---

Fig. 3 (*bottom left*). Effect of adding Mn^{++} on time courses of normal and tracer oxygen in the exchange reaction (Exp. 3). Vessel contents: 3 cc chloroplast suspension (containing 0.75 mg chlorophyll), 6 μM $MnCl_2$ in sidearm. To convert partial pressure units of the ordinate to microliters oxygen multiply by 1.53.

Fig. 4 (*bottom right*). Effect of Mn^{++} concentration on rates of oxygen production in the quinone reaction and on rates of net oxygen consumption of the subsequent quinone-stimulated Mehler reaction. Vessel contents: 3 cc chloroplast suspension (containing 0.5 mg chlorophyll), 6 mg catalase, 0.1 cc 50 % ethanol, 6 μM quinone, 0 to 8 μM $MnCl_2$.

TABLE I

EFFECTS OF ADDED MANGANOUS IONS ON RATES OF OXYGEN UPTAKE AND PRODUCTION IN MEHLER AND EXCHANGE REACTIONS

	MEHLER REACTION						EXCHANGE REACTION		
	EXP. 1			EXP. 2			EXP. 3		
	OXYGEN UPTAKE (μL/MIN)	OXYGEN PRODUCTION (μL/MIN)	NET RATE (μL/MIN)	OXYGEN UPTAKE (μL/MIN)	OXYGEN PRODUCTION (μL/MIN)	NET RATE (μL/MIN)	OXYGEN UPTAKE (μL/MIN)	OXYGEN PRODUCTION (μL/MIN)	NET RATE (μL/MIN)
Initial dark period	0	0	0	− 0.27	+ 0.01	− 0.26	0	0	0
Unstimulated light reaction	− 1.18	+ 0.44	− 0.74	− 1.64	+ 0.60	− 1.04	− 1.48	+ 1.34	− 0.14
Mn^{++} stimulated light reaction	− 2.27	+ 0.69	− 1.58	− 2.86	+ 0.74	− 2.12	− 1.98	+ 2.16	+ 0.18
Final dark period	0	0	0	0	0	0	0	0	0
% Increase in rate after addition of Mn^{++}	92 %	57 %	114 %	74 %	23 %	104 %	34 %	61 %	...

Vessel contents and experimental conditions: Exp. 1. 3 cc chloroplast suspension (containing 0.75 mg chlorophyll), 6 mg catalase, 0.1 cc 50 % ethanol, 6 μM $MnCl_2$ in sidearm; temp. 14.5° C. Exp. 2. 3 cc chloroplast suspension (containing 0.42 mg chlorophyll), other addenda the same as in Exp. 1; temp. 23° C. Exp. 3. 3 cc chloroplast suspension (containing 0.75 mg chlorophyll), 6 μM $MnCl_2$ in sidearm; temp. 14.5° C.

experimental data summarized in figure 4 show the effects of increasing amounts of manganous ions in reaction mixtures containing identical amounts of chloroplast suspension, quinone, catalase, and ethanol. Low concentrations of added Mn^{++} had no effect on either the rate of oxygen evolution in the quinone reaction or on the rate of net oxygen consumption in the Mehler reaction which followed quinone reduction. When amounts of Mn^{++} greater than 4 micromoles were added, however, the rates of oxygen production in the quinone reaction were reduced by approximately 20 %.

DISCUSSION

Stimulation of the Mehler reaction by manganous ions is similar in its effect on net oxygen uptake to the effects of quinone and ascorbic acid. Although net uptake of molecular oxygen is stimulated by all three substances, their effects on the actual rates of uptake and production of oxygen are different. These differences, then, must serve as the basis for any hypothesis concerning the nature of their action. In contrast to quinone, which stimulates the rates of uptake and production equally so that the stoichiometry of the over-all reaction remains unchanged (10), both ascorbic acid (11) and manganous ions produce an unequal stimulation of oxygen consumption and production with greater increments in the rates of oxygen uptake. Unlike the ascorbic acid effect, which lasts only so long as unoxidized ascorbic acid remains in the reaction mixture and in which the net amount of oxygen taken up in excess of the unstimulated rate is equivalent to the ascorbic acid initially added (11), there is no tendency for manganese-stimulated rates to revert to those of the controls. In this respect, manganese stimulation resembles that of quinone. Stimulated rates have been followed for several hours using the stable chloroplasts prepared from Phytolacca.

The catalytic nature of the manganese stimulation and the unequal stimulation of the rates of consumption and production in no way argue against a cyclic oxidation and reduction of manganese such as that proposed by Andreae (1) and also by Kenten and Mann (14). A chlorophyll-sensitized photooxidation of manganous ions superimposed on the Mehler reaction might be plausible via a scheme analogous to that proposed by Andreae (1) for his non-biological system. This is improbable, however, because of the observed enhanced rates of oxygen production. A more likely reaction mechanism would involve an alternate pathway mediated by Mn^{++} in the reaction sequence of the oxidized product of photolysis. Such a pathway could conceivably lead to oxygen production via peroxide formation. Under the conditions of the Mehler reaction, peroxide thus formed would be removed in the coupled oxidation of ethanol to acetaldehyde. A more efficient removal of the oxidized product would decrease the probability of a back reaction, or recombination of the products of photolysis. The enhanced reduction of molecular oxygen by the reduced product of photolysis could thus be a consequence of the more efficient removal of one of the products of photolysis. This reaction mechanism is consistent with all the experimental data.

Let us then examine the experimental results in terms of the proposed mechanism: Adding manganous ions to a Mehler reaction mixture resulted in increased rates of net oxygen uptake which could be

observed manometrically. Tracer studies showed that this enhanced rate of net uptake was the result of a stimulation of the rate of oxygen uptake which was considerably greater than the stimulation of its rate of production. In the presence of manganous ions, removal of the oxidized product of photolysis (other than by back reaction with the reduced product) could follow two possible pathways: i.e., the usual pathway to oxygen of the unstimulated Mehler reaction or peroxide formation mediated by manganous ions. An effective peroxide trapping mechanism (the catalase and ethanol in the Mehler reaction mixture) removes the peroxide thus formed. In accordance with the proposed mechanism then, uptake was enhanced and the ratio of uptake to production was greater than in the unstimulated reaction.

Adding manganous ions to chloroplast preparations with no other addenda (which on illumination carry on an oxygen exchange reaction) resulted once again in enhanced rates of both uptake and production of oxygen. In this case, the peroxide trap had not been added to the reaction mixture but the endogenous catalase of the chloroplasts was able to decompose peroxide catalatically by dismuting it to H_2O and O_2. The ratio of uptake to production should theoretically have remained constant because the alternate pathway for the oxidized product of photolysis results in oxygen production, as does the normal pathway. The calculated rates of oxygen uptake and production showed that their ratio changed from slightly more to slightly less than the theoretical value of one.

According to the proposed reaction mechanism, the presence of manganous ions in a Hill reaction mixture would not enhance the rate of oxygen production. Quinone seems to be efficient in its reaction with the reduced product of photolysis. Evidence for this is the observation that the rates of oxygen production with quinone as the oxidant are as a rule twice to several times those observed when oxygen is used as the Hill reagent. If a peroxide trap were present in the reaction mixture, however, we would anticipate that manganese-mediated peroxide production from the oxidized product of photolysis could decrease the rate of oxygen production. When catalase and ethanol were present during the quinone reaction and sufficiently high concentrations of Mn^{++} were added, a decrease in the rate of oxygen production was observed.

These experiments support the possibility that manganese-mediated peroxide formation is involved in the evolution of oxygen in photosynthesis. They do not, however, tell us whether oxygen evolution in vivo proceeds via peroxide formation similar to that which is indicated in vitro, or by the apparently normal pathway to oxygen which exists in unstimulated chloroplast preparations.

Summary

Tracer oxygen techniques were used to measure simultaneously the rates of consumption and production of oxygen in unstimulated and manganese-stimulated light-dependent chloroplast reactions. Both rates were stimulated on addition of manganous ions to the Mehler system and a similar stimulation of the related exchange reaction was observed.

These experiments indicate that the action of manganese is primarily involved with the oxidized product of photolysis. A reaction mechanism has been postulated whereby manganese mediates peroxide formation from the (OH)-product of the splitting of water. This provides an alternate pathway which results in the more efficient removal of this product and accounts for the stimulation of net oxygen uptake by manganous ions.

Acknowledgements

The author wishes to thank Prof. Allan H. Brown for his help and encouragement during the course of experimentation. These studies were aided through funds supplied by the Graduate School of the University of Minnesota and also by a contract between the Office of Naval Research, Department of the Navy and the University of Minnesota (NR 104 030). Tracer oxygen was obtained from Prof. A. O. C. Nier and was prepared under a grant from the American Cancer Society, through the Committee on Growth of the National Research Council. During the summer of 1955 this work was encouraged by the financial assistance of the Lawrence S. Moyer fellowship. It is with deep gratitude that this support is acknowledged.

Literature Cited

1. Andreae, W. A. 1955. The photoinduced oxidation of manganous ions. Arch. Biochem. Biophys. 55: 584–586.
2. Bishop, W. B. S. 1928. The distribution of manganese in plants and its importance in plant metabolism. Australian Jour. Exptl. Biol. Med. Sci. 5: 125–141.
3. Brown, A. H. and N. Good. 1955. Photochemical reduction of oxygen in chloroplast preparations and in green plant cells. I. The study of oxygen exchanges in vitro and in vivo. Arch. Biochem. Biophys. 57: 340–354.
4. Brown, A. H., A. O. C. Nier, and R. W. van Norman. 1952. Measurement of metabolic gas exchange with a recording mass spectrometer. Plant Physiol. 27: 320–334.
5. Brown, A. H. and D. Weis. 1959. Relation between respiration and photosynthesis in the green alga *Ankistrodesmus braunii*. Plant Physiol. 34: 224–234.
6. Emerson, R. and C. M. Lewis. 1940. The quantum efficiency of photosynthesis. Carnegie Inst. Washington Yearbook. 39: 154–158.
7. Gerretsen, F. C. 1949. Manganese in relation to photosynthesis. I. Carbon dioxide assimilation and the typical symptoms of manganese deficiency in oats. Plant and Soil. 1: 347–358.
8. Gerretsen, F. C. 1950. Manganese in relation to photosynthesis. II. Reduction-oxidation potentials of illuminated crude chloroplast preparations. Plant and Soil. 2: 159–193.

9. Good, N. and R. Hill. 1955. Photochemical reduction of oxygen in chloroplast preparations. II. Mechanisms of the reaction with oxygen. Arch. Biochem. Biophys. 57: 355–366.
10. Habermann, H. M. 1958. Light-dependent oxygen metabolism of chloroplast preparations. I. Stimulation following quinone reduction. Plant Physiol. 33: 242–245.
11. Habermann, H. M. and A. H. Brown. 1957. Certain effects of ascorbic acid on the reduction of oxygen in chloroplast preparations. In: Research in Photosynthesis, H. Gaffron, ed. Pp. 257–262. Interscience, New York.
12. Johnston, J. A. and A. H. Brown. 1954. The effect of light on the oxygen metabolism of the photosynthetic bacterium, *Rhodospirillum rubrum*. Plant Physiol. 29: 177–182.
13. Keilin, D. and E. F. Hartree. 1945. Properties of catalase. Catalysis of coupled oxidation of alcohols. Biochem. Jour. 39: 293–301.
14. Kenten, R. H. and P. J. G. Mann. 1955. The oxidation of manganese by illuminated chloroplast preparations. Biochem. Jour. 61: 279–286.
15. Kessler, E. 1955. On the role of manganese in the oxygen-evolving system of photosynthesis. Arch. Biochem. Biophys. 59: 527–529.
16. Kessler, E. 1957. Manganese as a cofactor in photosynthetic oxygen evolution. In: Research in Photosynthesis, H. Gaffron, ed. Pp. 243–249. Interscience, New York.
17. McHargue, J. S. 1922. The role of manganese in plants. Jour. Amer. Chem. Soc. 44: 1592–1598.
18. Mehler, A. H. 1951. Studies on reactions of illuminated chloroplasts. I. Mechanism of the reduction of oxygen and other Hill reagents. Arch. Biochem. Biophys. 33: 65–77.
19. Mehler, A. H. 1951. Studies on reactions of illuminated chloroplasts. II. Stimulation and inhibition of the reaction with molecular oxygen. Arch. Biochem. Biophys. 34: 339–351.
20. Mehler, A. H. and A. H. Brown. 1952. Studies on reactions of illuminated chloroplasts. III. Simultaneous photoproduction and consumption of oxygen studied with oxygen isotopes. Arch. Biochem. Biophys. 38: 365–370.
21. Pirson, A. 1937. Ernährungs-und stoffwechselphysiologische Untersuchungen an Fontinalis and Chlorella. Zeits. Bot. 31: 193–267.

COMPOSITION OF DEVELOPING PRIMARY WALL IN ONION ROOT TIP CELLS. I. QUANTITATIVE ANALYSES[1, 2]

WILLIAM A. JENSEN AND MARY ASHTON

DEPARTMENT OF BOTANY, UNIVERSITY OF CALIFORNIA, BERKELEY

An understanding of the factors influencing and directing cell development must rest on knowledge of the changes occurring during the course of cell development on both a morphological and biochemical level. Previous work on onion root tips has outlined some of these changes that occur early in the development of the cell (7, 8, 9, 11). The wall is one of the most conspicuous parts of the cell and undergoes great changes during the life of the cell. Very little is known, however, of the chemical composition of the wall during the early stages of formation. The analyses reported here attempt to enlarge the picture of the composition of the developing primary cell wall.

The root tip is an excellent material in which to study cell development, as the various stages are arranged in approximately linear order. Thus, the analysis of consecutive sections yields information on changes accompanying cell development. The cell develops rapidly, particularly during the early stages, and to follow the development, sufficiently thin sections must be used. The first 20 consecutive 100 μ sections of the onion root tip are analyzed in the present work.

The usual methods of cell wall analysis based on gravimetric measurements or on paper chromatography could not be used on this level. Lignin is not present in this region of the root and as all the other wall components can be analyzed in terms of carbohydrates (hexoses and pentoses) or simple carbohydrate derivatives (hexuronic acids), colorometric procedures were used. Microchemical techniques were developed to handle the material and measure the sugars. A standard extraction procedure of the wall components is used and the various extracts analyzed for the amount of hexuronic acids, hexoses, and pentoses present. The results are expressed as per cell and per unit surface area per cell.

MATERIALS AND METHODS

Sets of *Allium cepa* var. white globe were placed on vials of tap water and kept in the dark at 25° C. Roots between 1 and 2 cm in length developed in 48 hours. The first 5 to 10 mm of carefully selected uniform roots were excised and quickly frozen in isopentane cooled with liquid nitrogen. The root tips were then placed in methyl alcohol at −30° C. The methyl alcohol was changed once during the first 24 hour interval and three times during the second 24 hours, finally being replaced with toluene at the same temperature. After 2 hours the roots were brought to room temperature and the toluene changed. They were then paraffin infiltrated and imbedded. The roots were carefully aligned in groups of three during the imbedding.

The roots were sectioned transversely on a regular Spencer microtome at either 10 or 25 μ. As the analyses required 100 μ segments of the root, ten 10 μ sections or four 25 μ sections were collected and placed in a 1 ml centrifuge tube. Actually, when 10 μ sections were used only nine were placed in the tube, while the tenth was mounted on a microscope slide and stained. These sections acted as controls on the alignment of the roots and the condition of the tissue. The 10 μ sections were used in preliminary analyses while the 25 μ sections were used in the later replications. As the analyses required nine 100 μ segments, nine roots were cut and the identical sections placed in the same tube. This was facilitated during imbedding by aligning roots in groups of three in the paraffin.

Once the tissue was placed in the centrifuge tubes it was never removed. Changing the various liquids in the tubes without loss of tissue was a very critical step. Pipettes drawn from glass tubing were drawn a second time so that the opening was narrower in diameter than the smallest section. A holder rigidly supported the pipette which was connected to a flask and ultimately to a vacuum line. A stopcock in the top of the flask controlled the pressure in the flask and hence the rate of removal of the liquid. The centrifuge tube containing the tissue and solution was raised to the pipette by means of a small movable platform. A magnifying lens mounted in front of the tube, and a mirror mounted at one side aided in observing the tissue and in aligning the tube with the pipette. Care was taken to keep the tip of the pipette just below the surface of the liquid and to remove the liquid slowly. Where the liquid removed was to be saved, a rubber bulb with a small hole in the top replaced the vacuum line. By closing the hole with a finger, the bulb could be used to draw off the liquid. Opening the hole equalized the pressure in the bulb and the liquid remained in the pipette and was not carried up into the bulb. The same stands for pipette and tube were used as in the case of the vacuum line. The tubes in either case were usually centrifuged in a small micro

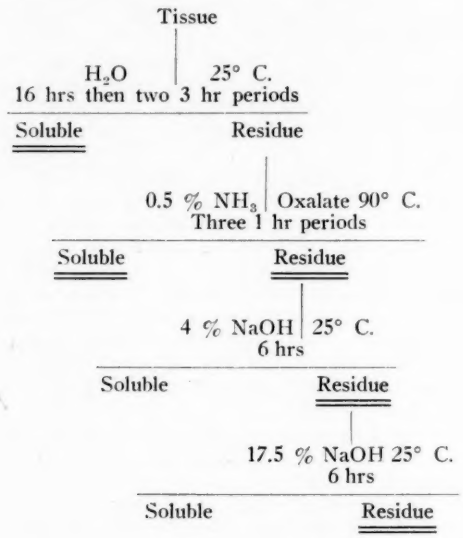
[1] Received July 16, 1959.

[2] Supported in part by a research grant from the National Science Foundation (G-4286).

centrifuge to force the tissue to the bottom before removal of the liquid.

The tubes were covered with aluminum foil wherever possible to reduce the amount of dust and lint falling into the tubes. Aluminum blocks 6in × 6in × 2in in which 100 holes 3/8in in diameter and 7/8in in depth had been drilled, were used for heating or cooling the tubes. These gave uniform heat conduction on a hot plate and could be cooled in a deep freeze.

The tissue was deparaffinized with toluene, rinsed with 100 % ethyl alcohol, and air dried. Analyses for total carbohydrate, hexoses, pentoses, and hexuronic acids were made directly on the tissue at this point, otherwise the sections were extracted according to the following schedule:



The extracts containing the soluble fraction were in each case combined and brought to dryness in an oven at 50° C. In the case of the 4 % NaOH extract, an equal volume of 4 % acetic acid was added to neutralize the base and then the solution evaporated. At all the underlined points in the above schedule, the extracts or residues were measured for amount of hexoses, pentoses, and hexuronic acids present. Of all the cell wall components fractionated in this scheme, only the 4 % NaOH and the 17.5 % NaOH soluble fraction had to be determined by difference in the residues before and after extraction, rather than directly on the extract. This was necessary when it was found that after treatment with NaOH neither the hexoses nor pentoses would react in the color reactions necessary for their determination.

This extraction schedule was worked out with great care. Longer and more numerous extraction periods were tested in most cases but the schedule given above proved to give the most satisfactory separation of wall components. Wherever possible alternate procedures were used to verify particular fractions. The ammonium oxalate extraction was replaced by extraction with 1*N* HCl and similar results were obtained. Balance sheets for the various carbohydrates were also run combining various extraction procedures.

The extraction procedure used here is similar to but not identical with that of Boroughs and Bonner (4) and differs from that of Jermyn and Isherwood (10) principally in not first extracting with 70 % alcohol and obtaining a cold water and ammonium oxalate fraction instead of a single hot water fraction. It also differs from that of Bishop, Bayley, and Setterfield (2) where a 10 % NaOH and a 17.5 % NaOH extraction are combined so that only one NaOH fraction was measured. Burström (5), who generally followed Jermyn and Isherwood also combined the alkali soluble fractions. A search of the literature revealed no two identical extraction schedules. This indicates that every material must be handled as a separate case.

The procedures listed below were used for the carbohydrate analyses. They are based on macro or semi-micro procedures (1,6), but in all cases have been further reduced in volume and modified.

I. Total Pentose and Hexose—Orcinal Reaction. To the sample was added 50 µl H_2O, 50 µl freshly prepared 2 % orcinal in 20 % H_2SO_4 and 500 µl 60 % H_2SO_4. The mixture was heated for 30 minutes at 80° C in an aluminum block and then cooled in a second, cold aluminum block. A "flea" of glass coated ferrum reductum was added and the solution stirred by moving the flea with a magnet. The absorption was measured at 425 mµ in a Beckman spectrophotometer model DU. The range of this procedure is 0.5 to 10 µg ± 0.1 µg. Glucose, fructose, galactose, mannose, ribose, and arabinose give equal absorption per µg, while xylose gives twice this absorption. Glucuronic and galacturonic acids give values of less than a tenth of the hexoses and pentoses while ascorbic acid gives no color at all. The orcinal reaction was used to measure the total combined hexoses and pentoses in the untreated tissue.

II. Hexuronic Acids—Carbazole Reaction. To the samples was added 50 µl H_2O and 300 µl concentrated H_2SO_4. The tubes were heated for 20 minutes at 100° C in an aluminum block and then cooled in a cold aluminum block for 5 minutes. Then 10 µl of a freshly prepared solution of 0.1 % carbazole in 95 % ethyl alcohol was added. A flea was used to stir the solution and the absorption measured at 535 mµ in a Beckman spectrophotometer. The procedure has a range of 0.5 to 5 µg ± 0.1 µg. Glucuronic and galacturonic acids give identical values. Pentoses and ascorbic acid do not react and there is negligible interference from hexoses. The carbazole reaction was used to measure hexuronic acid in the untreated tissue and in the various extracts.

III. Pentoses—Cysteine Reaction. The tubes containing the samples were cooled in a cold aluminum block and 50 µl cold H_2O and 200 µl cold concen-

trated H_2SO_4 were added. After shaking, the tubes were removed from the block, fleas added, and the tubes allowed to come to room temperature. The solutions were kept at room temperature for 2 hours with regular stirring. Then 5 μl of freshly prepared 3% aqueous cysteine was added and the mixture stirred. After 15 minutes, the absorption at 390 mμ and at 425 mμ was read in a Beckman spectrophotometer. The difference of absorption at 390 mμ to 425 mμ is proportional to the amount of pentose present. The range is 0.5 to 5 μg ± 0.1 μg. There was no reaction to hexoses, hexuronic acids, or ascorbic acid. The greatest difficulty with this procedure is that xylose gives 4.5 times the color per μg as does arabinose or ribose. In all cases the pentose data are expressed as arabinose. This cysteine reaction was used to measure pentoses in the untreated tissue, the extracts, and the tissue residues.

IV. Hexoses—Cysteine Reaction. To the sample was added 50 μl H_2O. The tubes were then placed in an ice water bath and 500 μl of a six part H_2SO_4 : one part H_2O mixture was added. After a few minutes the tubes were removed from the ice water, allowed to warm to room temperature, and then heated in boiling water for 3 minutes. They were then cooled in a water bath at room temperature and 100 μl 3% aqueous cysteine solution added. The solutions were stirred with a flea and the absorption at 415 mμ and 380 mμ read in a Beckman spectrophotometer. The difference in absorption at 415 mμ and 380 mμ equals the amount of hexose present. The range of the procedure is 0.5 to 5.0 μg ± 0.1 μg. Glucose, fructose, galactose, and mannose give almost identical absorption values. There is no interference from pentoses, hexuronic acids, or ascorbic acid. This cysteine reaction was used to measure hexoses in the untreated tissue, the extracts, and the tissue residue.

V. Total Hexoses and Pentoses—Indole Reaction. To the sample was added 50 μl H_2O, 500 μl 75% H_2SO_4 and 20 μl 1% alcoholic (95% ethyl alcohol) indole solution. This mixture was heated for 10 minutes at 100° C and cooled. The absorption was measured at 470 mμ in a Beckman spectrophotometer. The range of the procedure is 0.5 to 5.0 μg ± 0.1 μg for hexoses and pentoses. Glucose, fructose, mannose, galactose, arabinose, and ribose have equal absorption while xylose is 1½ times higher. Galacturonic and glucuronic acid react intensively while ascorbic acid gives less intense, but still appreciable, color. Consequently, the indole test was used only on the 4% and 17.5% NaOH tissue residue where neither the hexuronic acids nor ascorbic acid is present.

In all of the carbohydrate analyses two kinds of standards were used. In one the standards were treated the same as the tissue or extract and carried through the entire procedure. This standard acted as a control of the effect various chemicals and heating periods might have on the carbohydrates. The second standard was run only through the color reaction. In most cases there was little difference between the two standards.

The carbohydrate procedures given above were worked out in great detail. All hydrolysis times and temperatures were tested and complete absorption curves run in every case. Each procedure was tested for the possibility of interference by higher concentrations of sugars not normally reactive in that procedure. Even at ranges of more than five times the amount of the carbohydrate normally present in the tissue, no interference in any of the tests was found.

Attempts were made to remove the protoplasm from the cells before analysis of the wall by treatment with alcoholic KOH. These attempts were abandoned after determinations showed large losses of hexuronic acids, hexoses, and pentoses. As there is little hexuronic acid in the cell other than that associated with the cell wall and the total amount of pentoses found in all the nucleic acids in the cell give an insignificant color under the conditions used here, it was concluded that not only the protoplasm but much of the cell wall material was being removed. Onion root tips do not contain starch and it would appear safe to assume that the significant amount of the hexuronic acids, hexoses, and pentoses, is either associated with the cell wall or is soluble.

The roots were analyzed morphologically in the same way as reported earlier (9). A group of bulbs different from that used in previous work was used in this study. The roots from the new group of bulbs had slightly different cell numbers and cell dimensions. For these reasons the cell number, mean cell cross sectional area, mean cell length, and mean cell surface area are presented here. The number of mistosis also were counted.

Results

Morphology. The first 2 mm of the root tip are represented diagrammatically in figure 1. The graph immediately below the diagram presents the cell number per 100 μ section. The upper curve is the total cell number while the lower represents the number of cells per section minus the number of rootcap cells. Figure 1 presents the average cross sectional area, length, and surface area of the cells in each 100 μ section. The percent of nuclei in division is also presented in figure 1. A detailed morphological analysis of the onion root has been published (9) and complete discussion of the morphology of the root will not be undertaken here.

The general pattern of cell development can easily be seen from the graphs of figure 1. The cell, basal to the apical initials, first enters a period of marked radial enlargement. This period lasts from 400 to 1,400 μ from the tip. Elongation begins some 800 to 1,000 μ from the tip and extends beyond the 2 ml of the tip analyzed here. From 800 to 1,400 μ there is a period in which the cell is both enlarging radially and elongating. The amount of cell elongation, however, is small until after approximately 1,400 μ from

the tip. The period where elongation and radial enlargement overlap will be termed the first period or phase of elongation, while elongation after 1,400 μ will be referred to as the second phase. This pattern is in general agreement with that of Scott et al (11).

The distribution of cell divisions must also be considered in relation to the various stages of cell development. The apical initials are very low in number of cells in division. This feature of the root has been discussed elsewhere (9). During the period of radial enlargement, the number of cells in division increases and the maximum number of divisions occurs at the beginning of elongation. During the first phase of elongation, cell divisions are as numerous as during the first phase of radial enlargement. As the second phase of elongation is approached, cell divisions decrease and by 1,600 μ have ceased. This pattern of divisions is similar to that reported earlier by Jensen and Kavaljian (9). In the roots used by them, however, the rootcap was more extensive and the first 2 mm of the root analyzed, essentially did not contain the second period of elongation. Moreover, they used fluctuating temperatures while a constant, slightly elevated (25° C) temperature was used in the present work.

The cells of the rootcap in front of and surrounding the apical initials undergo rapid development resulting in large, heavy walled cells. The presence of the rootcap cells surrounding the apical initials must not be forgotten when considering the data from the early sections which contain large numbers of rootcap cells as well as the apical initials and other nonrootcap cells. The amount of carbohydrate present in the apical initials can be calculated if it is assumed that the amount of carbohydrate per rootcap cell immediately in front of the apical initials, is the same as in the rootcap cells surrounding the apical initials. Since the amount of carbohydrate in the rootcap cells in front of the apical initials is known from section II, and the number of rootcap cells present in section III is known from the morphological analysis, then simple multiplication of these two figures will result in the amount of carbohydrate in section III that will be accounted for by rootcap cells. If this figure is then subtracted from the total amount of carbohydrate in section III, the remainder will be the carbohydrate present in the apical initials. Dividing this figure by the number of apical initials, results in an estimate of the amount of carbohydrate present per cell of the apical initials. This figure is really only an approximation, but it is nonetheless interesting and useful.

CARBOHYDRATE DETERMINATIONS. The results of the carbohydrate determinations on the whole tissue and after the various extraction procedures, are presented graphically per cell and per μ^2 surface area per cell, in figures 2 and 3. All data have been expressed per μM or per $\mu\mu$M so that the data on the various sugars can be compared directly.

Before describing the results a word must be said regarding terminology. Two general systems of cell wall terminology are apparently in existence. One is based on solubility criteria so that all hot water or ammonium oxalate soluble materials become pectin

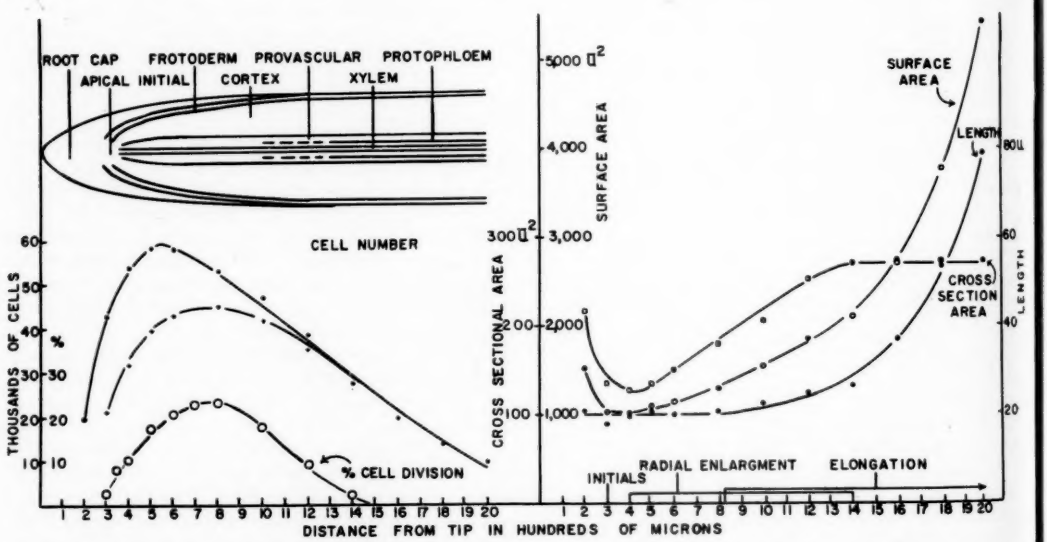


FIG. 1 (*left graph*). Number of cells per 100 μ section. (· = total number of cells per section, + = nonrootcap cells per section) and percent cells in division. The diagram of the root at the top is on the same scale as the graph.

(*Right graph*) Average cell cross section area, length, and surface area expressed as a function of distance from tip of section analyzed.

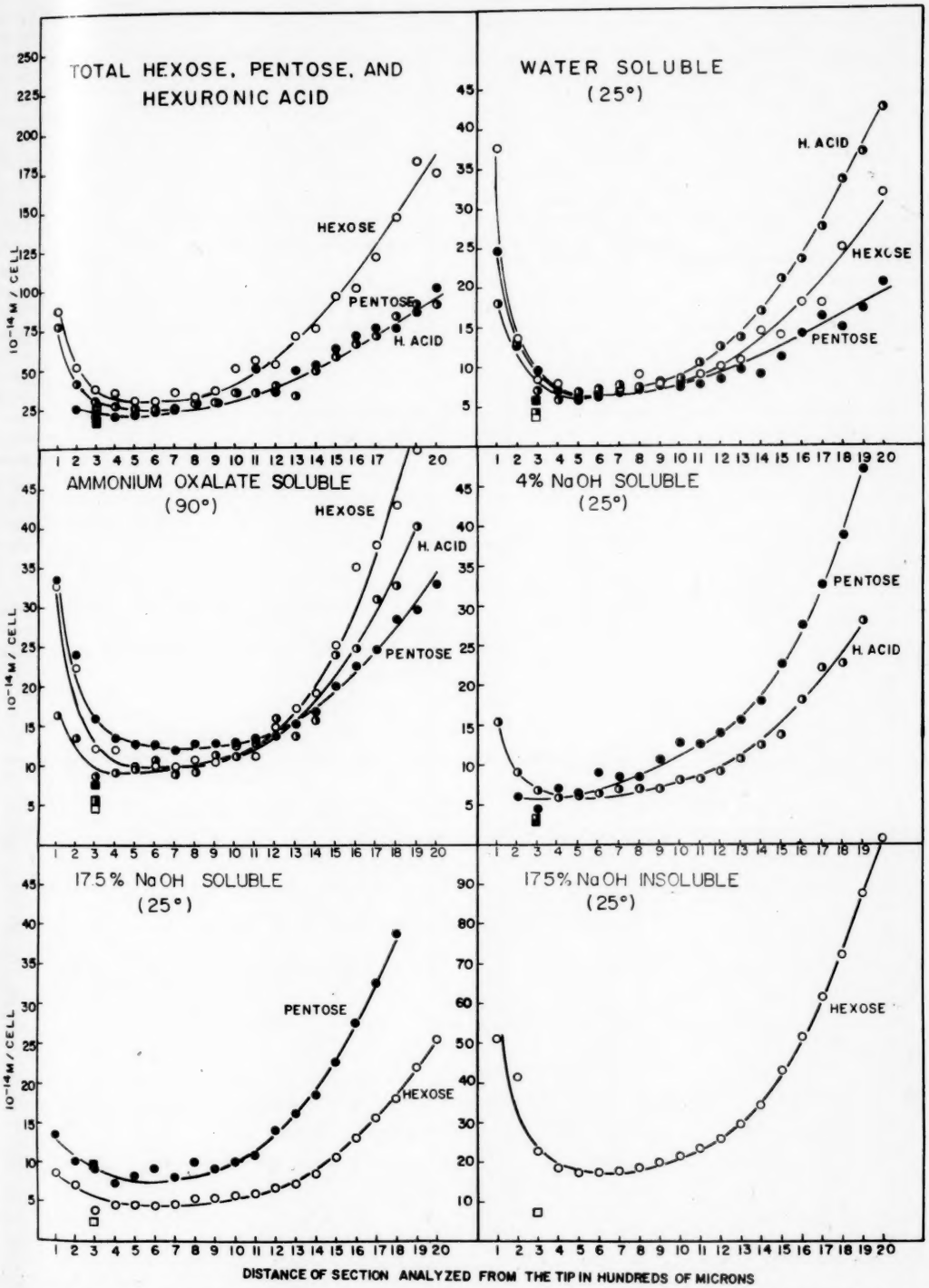


FIG. 2. Results of carbohydrate analyses expressed per cell. Open circle is hexose; closed circle is pentose; half-open circle is hexuronic acid. The squares indicate calculated values for the apical initials (see text for explanation).

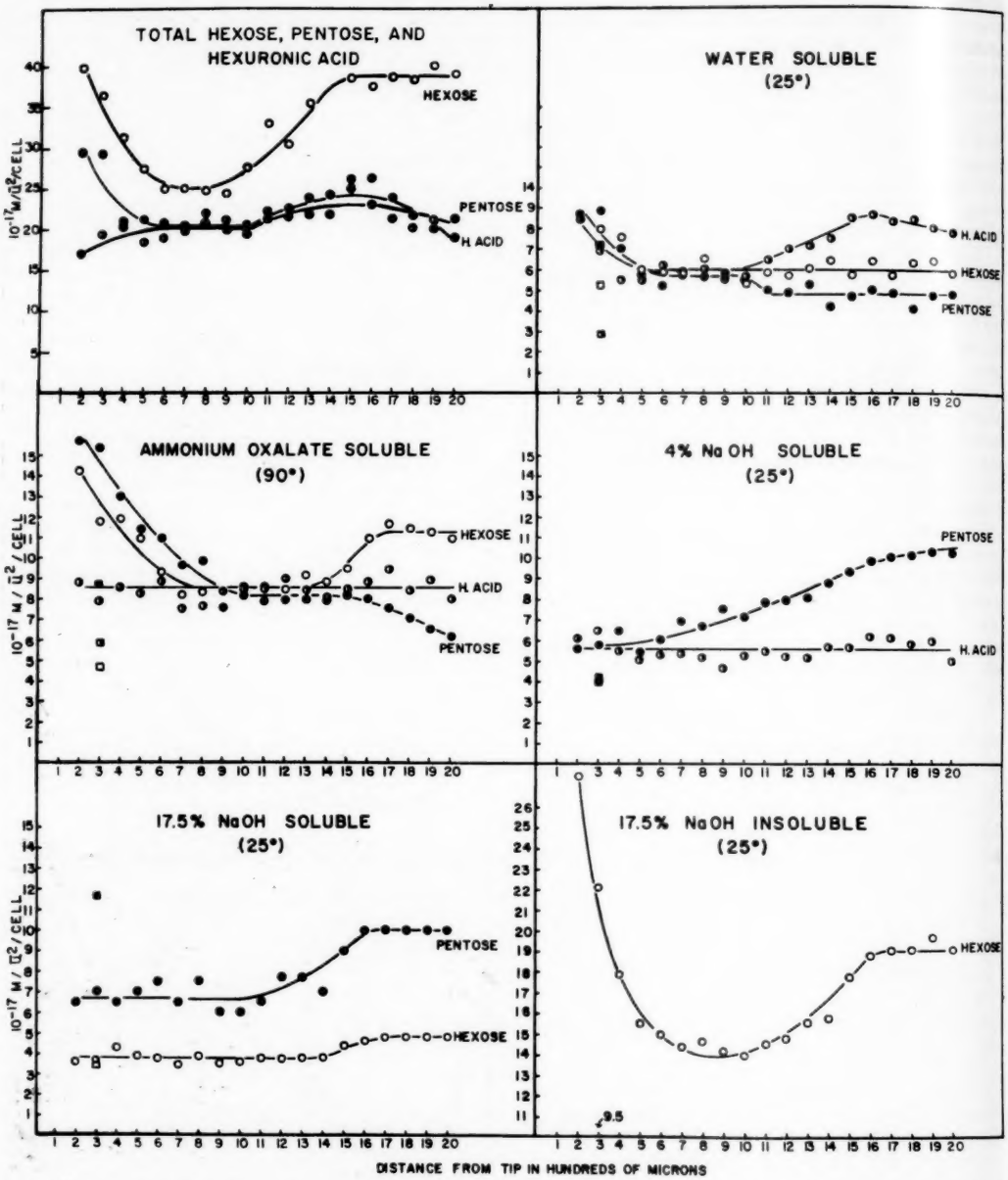


FIG. 3. Results of carbohydrate analyses expressed per surface area per cell. Open circle is hexose; closed circle is pentose; half-open circle is hexuronic acid. Squares indicate calculated values for apical initials.

and alkali soluble materials become hemicelluloses (10). The other is based on chemical composition criteria so that only pure polygalacturonic acid compounds are pectic substances, while hemicelluloses contain hexuronic acid and pentose, and noncellulosic polysaccharides composed of hexoses and pentoses may be found either in the hot water-ammonium oxalate soluble or alkali soluble fraction (3). This picture is further complicated by dividing the pectic substances (in a chemical sense as polygalacturonic acid) into pectin and protopectin on solubility characteristics. Only cellulose and lignin are clearly uncontroversial terms.

The following definitions will be used: pectin—water soluble hexuronic acids; protopectin—water insoluble, ammonium oxalate soluble hexuronic acids; pectic substances—the sum of the water and ammonium oxalate soluble hexuronic acids; soluble non-cellulosic polysaccharides—water insoluble, ammonium oxalate soluble hexoses and pentoses; hemi-cellulose—ammonium oxalate insoluble, 4 % NaOH soluble hexuronic acids and pentoses; insoluble non-cellulosic polysaccharides—4 % NaOH insoluble, 17.5 % NaOH soluble hexoses and pentoses; cellulose—17.5 % NaOH insoluble hexose.

Quantitative comparison of the present data with previous work is almost impossible. This is because the present data is expressed per cell and almost no data on a similar basis exist in the literature. Moreover, it is not possible to obtain the dry weight of the sections making it impossible to express the present data in terms of percent dry weight as is common in the literature.

To understand the implications of the data expressed per unit surface area per cell it is necessary to realize that there need be no connection between the surface area of the cell and the area of the wall. When the data are expressed per unit area they mean that the amount of wall material per cell is being compared with the surface area of the cell. If the amount of wall material and the area of the cell both increase uniformly from one section to another the amount of wall material per unit area will remain constant; if the wall materials increase more than the surface area, the amount of wall material per unit area will increase. There is no asumption that the wall is continuous or entire. From figure 4, it is clear, that except for the cell in rapid elongation, the wall is not a simple, entire sheet surrounding the cell. When the unit area data are viewed in this way and the factors involved in changes in surface area are examined, a picture of cell wall development can be outlined.

The important points of the determinations can be summarized thus:

A. Total Hexuronic Acid, Hexose, and Pentose. The three major groups of carbohydrates present in the root tip show, in the analysis of the total tissue, a unity and relationship that belies the diversity of their distribution within the cell wall. The cells of the root cap are high in both pentoses and hexoses while relatively low in hexuronic acids. All three groups are relatively low in the cells undergoing radial enlargement and increase proportionally to the increase in surface area of the cell. Correlated directly with the beginning of elongation there is an increase in amount of all three groups of carbohydrates. This increase in amount is greater than the increase in surface area of the cell. As the cells enter the second phase of elongation, the amount of hexuronic acids, pentoses, and hexoses continues to increase, but only the hexoses continue to increase at a rate greater than the increase in surface area.

These determinations of total hexuronic acid, hexose, and pentose were also used as a check on the measurement of the various fractions. When the hexuronic acid, hexose, and pentose content of the various fractions are added together they should equal the total content of these carbohydrates measured directly. The direct and indirect total amounts were found to differ by less than 3 % for the hexoses and less than 8 % for the hexuronic acids. The pentose difference was almost 30 %, however, with the indirect total being higher than the direct. The pentoses are the most difficult to measure of the three carbohydrates, requiring a procedure that does not allow the tissue to be heated. This fact makes it highly probable that the direct total amount is lower than the indirect total because of difficulties in obtaining complete hydrolysis of the wall.

B. Water Soluble Fraction. All three groups of carbohydrates were found in the water soluble fraction in relatively large amounts. As the tissue was in contact only with toluene or absolute methyl or ethyl alcohol from the time of initial freezing until the water extraction, we believe that no measurable amount of carbohydrate was lost.

The hexuronic acids may compose part or all of the water soluble pectins. The cells of the root cap

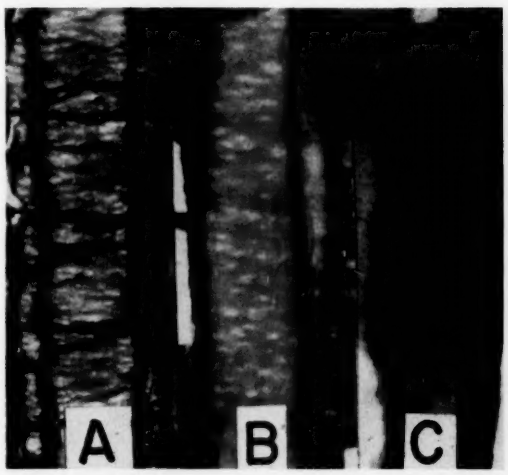
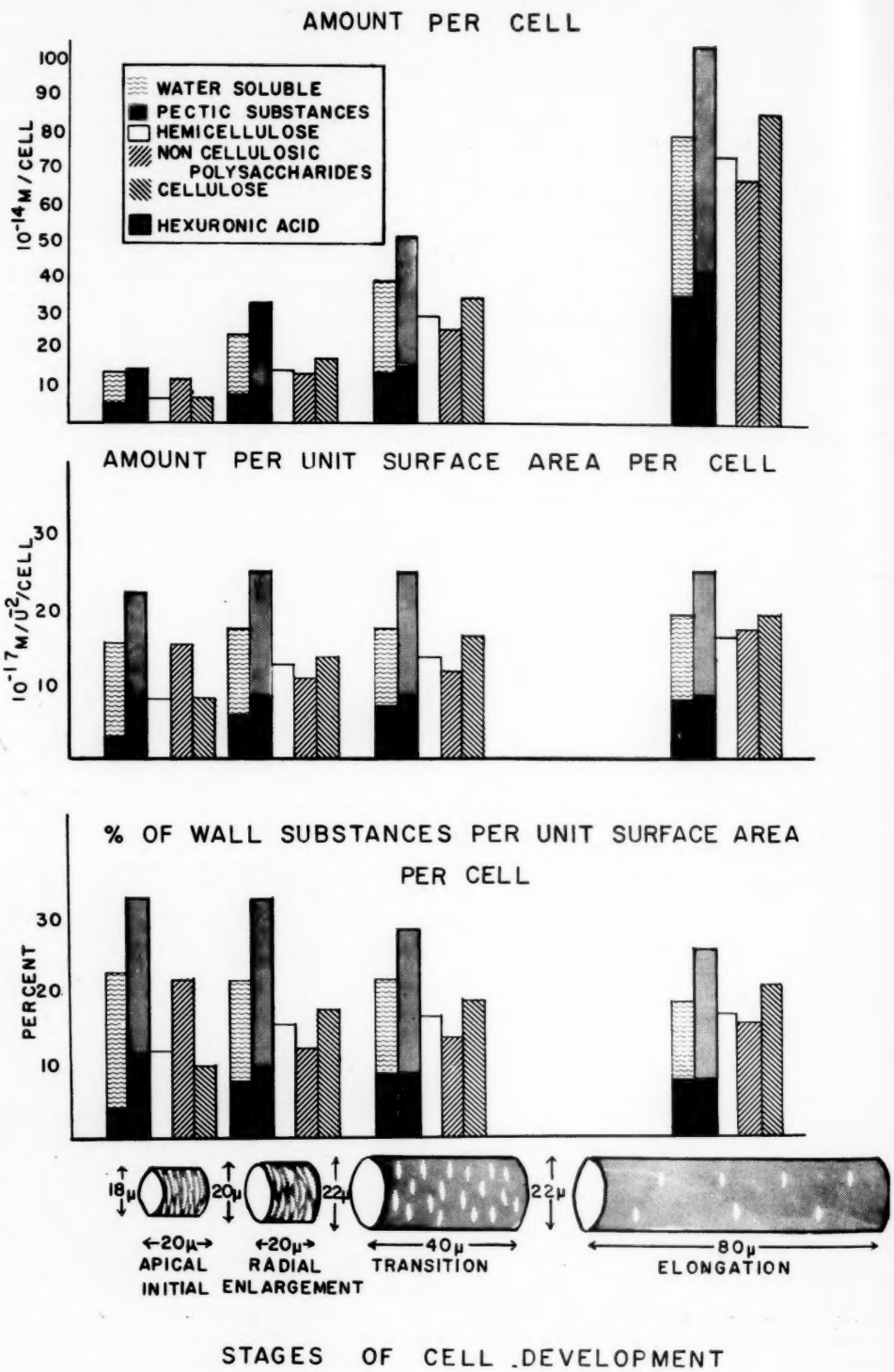


Fig. 4. Microscopic structure of cell wall. A. Wall of cells in radial enlargement. B. Wall of cells in transition zone. C. Wall of rapidly elongating cells.


AMOUNT PER CELL
WATER SOLUBLE
PECTIC SUBSTANCES
HEMICELLULOSE
NON CELLULOSIC POLYSACCHARIDES
CELLULOSE
HEXURONIC ACID
10^{-14} M/CELL
100
90
80
70
60
50
40
30
20
10
AMOUNT PER UNIT SURFACE AREA PER CELL
10^{-17} M/U^2/CELL
30
20
10
% OF WALL SUBSTANCES PER UNIT SURFACE AREA PER CELL
PERCENT
30
20
10
18μ
20μ
22μ
22μ
←20μ→ APICAL INITIAL
←20μ→ RADIAL ENLARGEMENT
←40μ→ TRANSITION
←80μ→ ELONGATION
STAGES OF CELL DEVELOPMENT

are relatively high in hexuronic acid. The cells in radial enlargement contain a relatively low amount which appears to be directly proportional to the surface area of the cell. As soon as elongation begins, however, the amount of hexuronic acid increases at a rate greater than the increase in surface area. As the cell reaches the second phase of elongation, the rate of increase of the hexuronic acid falls below the rate of increase of surface area and the amount of hexuronic acid per unit area progressively declines.

Hexose content of the root cap cells is also high, particularly in the extreme cells. In the cells in radial enlargement the amount of hexose is lower than in the rootcap. As cell elongation begins, hexose content per cell increases. This increase, however, is directly proportional to the increase in cell surface area so that the amount per unit area remains constant.

The pentose content per cell is similar to the hexose pattern except that at the beginning of elongation there is a small but consistent drop in pentose amount per unit area.

C. 0.5 % Ammonium Oxalate Soluble. Again, all three groups of carbohydrates were found and in relatively large amounts.

The hexuronic acids, which may be considered the protopectins and the pectates, are present in amounts directly proportional to the cell surface area in all stages of development represented in the first 2 mm of the root including the rootcap. The protopectins make up 6 % of the total carbohydrate content of the first section and increase to 10 % by the eighth section and then remain relatively constant. The sum of the pectin and protopectin follows a similar pattern, starting at 13 %, increasing to 18 % and then remaining constant.

Hexoses, probably in the form of a non-cellulosic polysaccharide, are present in large amounts in cells that have undergone or are undergoing cell elongation. The cells of the rootcap appear very high in hexoses and undoubtably influence the amount per unit area between 500 to 800 μ from the tip. During radial enlargement the amount of hexose is relatively low and directly proportional to cell surface area. This proportionality is maintained during early elongation but as the cells enter the second phase of elongation there is a sharp rise and a new proportionality at a higher level is established.

Pentoses, probably in the form of a non-cellulosic polysaccharide, are present in large amounts in the cells of the rootcap. Cells in radial enlargement and early elongation contain relatively less pentoses but the amount is directly proportional to the increase in surface area. As the rate of elongation increases, the increase in pentose content does not remain proportional but decreases so that the amount of pentose per unit area of the wall decreases.

D. 4 % NaOH Soluble Fraction. This fraction contained only hexuronic acid and pentoses and thus fits one commonly accepted definition of the polyuronide hemicelluloses (3).

The hexuronic acid content per cell is low in the cell in radial enlargement and increases as the cells enter elongation. In all phases of cell development the amount per cell is directly proportional to surface area.

Pentose content per cell is directly proportional to surface area only during the very early stages of cell development. During this period the ratio of pentose to hexuronic acid is 1 : 1. While the cells still are undergoing radial enlargement, the amount of pentose per cell begins to increase at a rate greater than the increase in cell surface area. This condition persists until the second phase of elongation when the amount of pentose decreases slightly in relation to increase in surface area of the cell.

This pattern of hexuronic acid and pentose change suggests that the hemicellulose present consists of chains of hexuronic acid-pentose units in a ratio of 1 : 1. The extra pentose molecules may form cross linkages between the hexuronic acid-pentose chains.

E. 17.5 % NaOH Soluble. This fraction contained only hexose with an occasional trace of pentose and represents almost pure cellulose.

The hexose content of the rootcap is high while the cells in radial enlargement are relatively low. As cell elongation begins, the hexose content of the wall begins to increase at a rate greater than the increase in surface area. When cell elongation enters the second phase, however, the rate of increase of hexose per cell becomes constant in relation to the increase in surface area.

Discussion

The composition of the primary cell wall in the root tip presents a surprisingly complicated picture. The problem is to relate the changes in the chemical composition of the wall with the morphological development of the cell. The region of the root analyzed contains cells of the rootcap, the apical initials, and cells in the following stages of morphological development: radial enlargement, a transition stage where radial enlargement and elongation are both going on, and elongation. The data for the apical initials and the various stages of cell development are summarized in figure 5.

Fig. 5. Composition of cell wall expressed as a function of stages of development of cell. The noncellulosic polysaccharides referred to in the legend are the insoluble noncellulosic polysaccharides of the text. The hexuronic acid content of the water soluble and pectic substances fraction are shown because of importance of the hexuronic acids in definitions of pectin and protopectin. The black bars in both cases represent the amount of hexuronic acid in each fraction.

The cells of the rootcap actually are present in all stages of development but the mature, fully differentiated cells predominate. These cells are large and possess very thick walls. From the chemical analyses it is clear that the walls contain large amounts of cellulose and soluble non-cellulosic polysaccharides. The cells also contain fairly large amounts of water soluble hexoses, pentoses, and hexuronic acids. Protopectin, hemicellulose, and the insoluble non-cellulosic polysaccharides are present in much lower amounts and are directly proportional to cell surface area.

The apical initials represent a relatively small group of cells that can only be treated indirectly on the basis of calculations previously described. The striking feature of the apical initials is their low incidence of division. They appear to be metabolically inactive insofar as they have been examined. Morphologically, the apical initials are small cells with thin, delicate appearing cell walls that in no sense form a continuous sheet of material. On a per cell basis, the apical initials are low in all cell wall components, except the insoluble pentose non-cellulosic polysaccharides, when compared with the other cells in the root (fig 5). On a per unit surface area basis, however, all the pentose containing fractions, except the pentose in hemicellulose, are either equal to or greater than the same fractions in the cells in radial enlargement. Pectin and protopectin are low on either basis but the wall is proportionally richer in protopectin than is the wall in radial enlargement. Cellulose is also low but the proportion of cellulose to pectic substances is the same in the apical initials and the cells in radial enlargement. It is clear that the cells of the apical initials do not lack cellulose, nor are very high in pectic substances or composed only of pectic substances and cellulose.

The cells in radial enlargement are increasing primarily in diameter with little increase in average length. Cell divisions are numerous during this stage and increase in number. The wall is morphologically a loose open structure with large gaps (the primary pit fields) running at right angles to the long axis of the cell (fig 4a). The wall is low in all carbohydrates although all components are present in roughly equal amounts. If all the water and ammonium oxalate soluble carbohydrates are taken together and treated as pectic substances they amount to some three times the amount of cellulose present. If, however, only the hexuronic acids of these fractions are considered as pectic substances they are then equal to the cellulose present. During this period of radial enlargement all the wall components increase uniformly in relation to one another and in direct proportion to the increase in cell surface area. The cells in radial enlargement are often considered as the meristem and treated as being simple meristematic cells. In this connection it should be emphasized again that the wall does not lack cellulose nor is it composed primarily of pectic substances.

Radial enlargement continues until approximately 1,400 μ from the tip, while elongation begins some 800 to 900 μ from the tip. Thus, there is a region where the cell is still increasing in diameter while at the same time beginning to increase in length. Cell division in the root reaches a peak at the beginning of this stage and decreases as the stage progresses and elongation becomes more pronounced. During this stage the morphology of the wall changes to a more continuous structure with the larger gaps being filled in, although still visible, and the primary pit fields becoming smaller (fig 4b). This change in the morphology of the wall is reflected in the amount and composition of the various wall components. All the carbohydrate components of the wall increase during this period but three (pectin, soluble pentose noncellulosic polysaccharide, and cellulose) increase more than the surface area of the cell.

As radial enlargement ends and several hundred microns later cell divisions cease, the cell enters the stage of rapid elongation. The morphology of the wall changes again, becoming continuous with all but the final traces of the primary pit fields nearly invisible (fig 4c). The change to straight elongation has some very interesting effects on the carbohydrates of the cell wall. All components continue to increase per cell but it is in relation to surface area that surprising changes occur. From 1,600 to 2,000 μ from the tip a steady state seems to exist between the increases in cell wall components and the increase in cell surface area (all cell wall components increase directly proportional to surface area). This steady state has been arrived at differently, however, for the different components. In the case of protopectin and hemicellulosic hexuronic acid no change was involved as they remained directly proportional to surface area through all the stages of development. For cellulose and pectin which were from the beginning of elongation increasing faster than the surface area, the final state is a result of synthesis now becoming directly proportional to growth in area. For soluble hexose noncellulosic polysaccharides and the insoluble noncellulosic polysaccharides, the end of radial enlargement resulted in a period of increased synthesis that terminated in the establishment of a new proportionality to surface area but at a higher level. In all cases the end result is a condition where each component is being synthesized in direct proportion to the increase in surface area of the cell. The composition of the wall is different, however, than it was before. Per unit area, the wall is higher in cellulose, pectin, hemicellulose, soluble hexose noncellulosic polysaccharide, and the insoluble noncellulosic polysaccharides while remaining constant with regard to protopectin and water soluble hexose. Only water soluble pentose decreased in amount.

What essentially appears to be happening during this stage of development is that the gaps in the cell wall associated with radial enlargement are being filled in with cellulose, pectin, hemicellulose, soluble hexose noncellulosic polysaccharide, and the insoluble noncellulosic polysaccharides. This accounts for the increase of these components per unit area immediately after the cessation of radial enlargement and for the

fact that they then become directly proportional to surface area at a higher level while the wall appears no thicker, if not thinner, morphologically. When this stage is compared with the preceding one where both radial enlargement and elongation are occurring simultaneously, it seems possible that the amount of elongation in the earlier stage may be limited by the fact that the wall is still too fragmentary for rapid elongation to occur. Only after the wall is an approximately continuous unit, can elongation occur to any great extent. This point of conversion would appear to be a logical place to study the effect of growth substances on roots.

The data presented here are difficult to compare with other cell wall analyses due to differences in the techniques and the means of expression employed. Insofar as comparison is possible the data reported here are in general agreement with other analyses of primary walls using quantitative biochemical techniques (3, 4, 5, 10). The notable exception is the work of Bishop et al (2), who reported small amounts of pectic substances in the primary wall of *Avena coleoptiles*. The striking difference in the materials may, however, very well account for the differences in the results. We agree with Bishop et al in the conclusion that the hemicelluloses and noncellulosic polysaccharides may be more important in cell wall development than generally recognized.

Summary

The first 20 consecutive 100 μ sections of the onion root tip were analyzed for water soluble, 0.5 % ammonium oxalate soluble, 4 % NaOH soluble, 17.5 % NaOH soluble, and 17.5 % NaOH insoluble hexose, pentose, and hexuronic acids. The data were expressed per cell and per μ^2 surface area per cell.

Besides the rootcap and apical initials, this region of the root contains the following stages of cell development: radial enlargement, a transition stage where both radial enlargement and elongation occur. and elongation. On the basis of the present analysis the cell wall in these stages can be characterized. The rootcap cell is high in cellulose and soluble noncellulosic polysaccharides, fairly high in water soluble hexoses and pentoses and pectin, and low in protopectin, hemicellulose, and insoluble noncellulosic polysaccharides. The apical initials were low in all cell wall components although all were present, the pectic substances and cellulose being present in equal amounts. In radial enlargement the wall was still low in all components but the amounts of the major components were approximately equal. During the transition stage all components increased per cell while on a per unit area basis the only component that showed an increase that could be correlated with the beginning of elongation was pectin. When radial enlargement ceased there was a marked increase in the amount of cellulose, pectin, soluble hexose noncellulosic polysaccharide, and insoluble noncellulosic polysaccharides per unit area. This increase can be correlated to morphological changes in the wall. During the stage of elongation all components increased in direct proportion to the increase in surface area of the cell.

Literature Cited

1. Ashwell, G. 1957. Colorimetric analysis of sugars. In: Methods in Enzymology, S. P. Colowick and N. O. Kaplan, eds. Academic Press, New York.
2. Bishop, C. T., S. T. Bayley, and G. Setterfield 1958. Chemical constitution of the primary cell walls *Avena coleoptiles*. Plant Physiol. 33: 283–289.
3. Bonner, J. 1950. Plant Biochemistry. Academic Press, New York.
4. Boroughs, H. and J. Bonner 1953. Effects of indoleacetic acid on metabolic pathways. Arch. Biochem. Biophys. 46: 279–290.
5. Burström, H. 1958. The influence of growth regulators on the composition of the cell wall. Fysiogr. Sällskapet. Forhandl. I. Lund 28: 53–64.
6. Dische, Z. 1955. New color reactions for the determination of sugars in polysaccharides. In: Methods of Biochemical Analysis, D. Glick, ed. Vol. 2. Interscience Publishers, New York.
7. Jensen, W. A. 1958. The nucleic acid and protein content of root tip cells of *Vicia faba* and *Allium cepa*. Exp. Cell Research 14: 575–583.
8. Jensen, W. A. 1958. Carbohydrate content of the root tip cells of *Allium cepa*. Plant Physiol. 33: 64–65.
9. Jensen, W. A. and L. G. Kavaljian 1958. An analysis of cell morphology and the periodicity of division in the root tip of *Allium cepa*. Amer. Jour. Bot. 45: 365–372.
10. Jermyn, M. A. and F. A. Isherwood 1956. Changes in the cell wall of the pear during ripening. Biochem. Jour. 64: 123–132.
11. Scott, F. M., K. Hamner, E. Baker, and E. Bowler 1956. Electron microscope studies of cell wall growth in onion root. Amer. Jour. Bot. 43: 313–324.
12. Whaley, W. G., L. W. Mericle, and C. Heimsch 1952. The wall of the meristematic cell. Amer. Jour. Bot. 39: 20–26.

DIFFERENTIAL UPTAKE OF 2,4-D ACID AND ITS OCTYL ESTER BY SEEDLING CORN ROOTS AND COLEOPTILE SECTIONS[1, 2]

D. JAMES MORRE AND B. J. ROGERS

PURDUE UNIVERSITY, LAFAYETTE, INDIANA

Although much research has been directed toward the uptake of inorganic ions by roots, the entry of organic compounds has received little attention. The present study concerns a basic difference in permeability between seedling corn roots and coleoptile sections as indicated by a differential uptake of ionized 2,4-D acid and the apolar octyl ester of 2,4-D.

It is known that the addition of an alcohol through an ester linkage drastically changes the physical properties of the 2,4-D molecule (5). In any study of the effect of molecular changes on toxicity, it is important to determine whether the changes, if any, affect the inherent toxicity or merely affect the ability of the molecule to reach the site of activity. It seemed possible that the transformed dosage-response curve, as applied to spore toxicity by Rich and Horsfall (6), could provide this information.

The inhibition of primary root growth was determined for corn seedlings grown in buffered solutions which contained graded concentrations (on an acid equivalent basis) of either 2,4-D acid or its octyl ester. The experiments were conducted at pH 7, with a 24 hour incubation period. The difference in slopes of the resultant lines established by linear regression was not statistically significant, implying that the inherent toxicity of the 2,4-D molecule is not changed by esterification. However, a large difference was found for the ED_{50} (concentration necessary for 50 % inhibition of root growth) i.e., 4.5 mg/l for 2,4-D and 9.7 mg/l for the octyl ester. Theoretically the ED_{50}'s should reflect the ability of the toxicant to reach the site of activity (6) since the mode of action is unchanged.

MATERIALS AND METHODS

Uptake of the acid and ester was measured directly. The octyl ester was synthesized by means of the acid chloride from 2,4-D-2-C^{14} obtained from Nuclear-Chicago Corp. Both the final 2,4-D solution and the ester emulsion were of the same concentration (500 mg/l acid equivalent) and specific activity (2,200 cpm/ml). The solutions were prepared in 0.01 M sodium phosphate buffer at pH 7 and 0.05 % Tween 80. Corn seedlings germinated 72 hours were grown on filter papers in Petri plates containing 5 ml of the solution to be tested. At intervals of 1, 2, and 3 hours seedlings were removed, washed in ethanol, and prepared for counting. The roots were ground in a mortar, transferred to a planchet, dried, and the radioactivity counted directly at infinite thickness. Due to the low specific activity in the remainder of the seedling, the seeds and shoots were ground and extracted with ether. The ether extract was evaporated directly on the planchet. A Baird Atomic automatic sample changer with a detector of the Sugarman type was used with a pre-set count to provide 1 % accuracy.

Corn coleoptile sections, prepared similarly to the Avena coleoptiles of Johnson and Bonner (4), were floated in 20 ml of the same solutions as used for the root studies. Twenty sections were removed at intervals of 1, 2, and 3 hours, washed in ethanol, dried in the planchet, and counted directly as above.

RESULTS AND DISCUSSION

The curves for uptake of 2,4-D acid and ester for both intact roots and coleoptile sections (figs 1 and 2), follow those of Johnson and Bonner (4) quite closely. Severe growth inhibition appeared between 3 and 6 hours after treatment. Radioactivity appeared in the seeds and shoots in the same ratio as the roots. From measurements of radioactivity in the plant tissue and inhibition of root growth, it was apparent that the quantity of octyl ester entering the roots was considerably less than that of the acid. This same result was observed with intact cucumber roots and excised corn roots. On the other hand, more radioactivity from the octyl ester appeared in coleoptile sections; increased growth of coleoptile sections also indicated a greater uptake of the octyl ester. Uptake of the ester can be attributed partially to an in vivo conversion to the acid. Such a conversion would maintain the concentration gradient of 2,4-D ester in favor of continued uptake. Plant enzymes have been shown to catalyze the de-esterification. The results of this work will be published elsewhere.

[1] Received July 22, 1959.

[2] Contribution from the Department of Botany and Plant Pathology, Purdue University, Lafayette, Ind. Journal Paper No. 1415, Purdue University Agricultural Experiment Station.

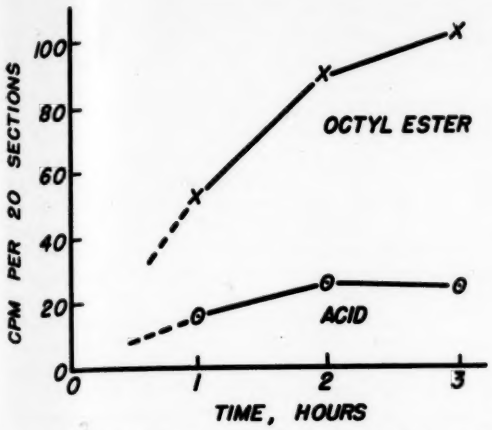


FIG. 1. Uptake of 2,4-D-2-C^{14} and 2,4-D-2-C^{14} octyl ester by intact corn roots. Data include radioactivity measurements for the entire seedling so that total uptake was measured. Each point represents an average of three replications of ten corn seedlings grown under uniform conditions.

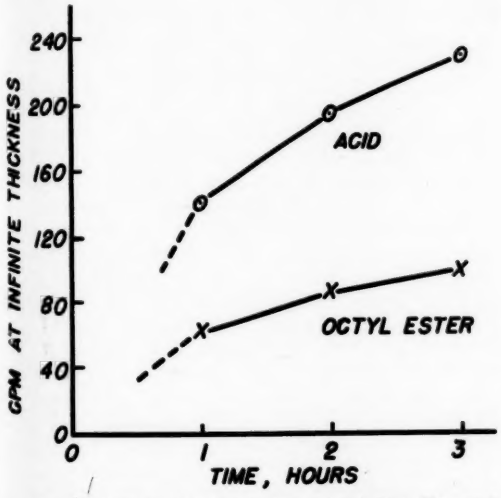


FIG. 2. Uptake of 2,4-D-2-C^{14} and 2,4-D-2-C^{14} octyl ester by excised corn coleoptile sections. Each point represents an average of duplicate counts from 12 replications of 20 coleoptile sections taken from corn seedlings grown under uniform conditions.

It might be argued that the observed differences in uptake are due to the fact that the ester was supplied as an emulsion. This interpretation is discounted since the slope of the line representing 2,4-D ester inhibition of corn roots is not changed through a range of concentrations which involved both true solutions and finely divided emulsions stabilized by the addition of Tween 80. Spontaneous hydrolysis undoubtedly occurs but apparently has no major effect on the results. It should be recognized that the acid is near complete ionization at pH 7 (7) but the ester is still less effective than the acid in inhibiting root growth at pH 5.6, the lowest pH tested.

When the relative solubilities of 2,4-D acid and ester in water and organic solvents are considered (5), the measurements of uptake by roots are not in agreement with the Overton-Meyer theory but confirm the suggestion of Crafts (3) that roots are more adapted to the uptake of ionized compounds. Conversely, results with coleoptile sections are consonant with the classical view that uptake of the apolar ester should be greater than for the ionized acid (2).

It is interesting to note in this regard that Åberg (1) demonstrated that the activity of the methyl ester of 2,4-D in inhibiting the growth of flax roots was higher than the acid. This suggests that the observations reported here may apply only to esters with high oil-water partition coefficients.

LITERATURE CITED

1. ÅBERG, B. 1950. On auxin antagonists and synergists in root growth. Physiol. Plantarum 3: 447–461.
2. CRAFTS, A. S. 1957. The chemistry and mode of action of herbicides. In: Advances in Pest Control Research, R. L. Metcalf ed. Vol. I. Pp. 39–79. Interscience, N. Y.
3. CRAFTS, A. S. 1948. A theory of herbicidal action. Science 108: 85–86.
4. JOHNSON, M. P., and J. BONNER. 1956. The uptake of auxin by plant tissue. Physiol. Plantarum 9: 102–118.
5. NEX, R. W. and A. W. SWEZEY. 1954. Some chemical and physical properties of weed killers. Weeds 3: 241–256.
6. RICH, S., and J. G. HORSFALL. 1952. The relation between fungitoxicity, permeation, and lipid solubility. Phytopathology 42: 457–460.
7. WEDDING, R. T., L. C. ERICKSON, and B. L. BRANNAMAN. 1954. Effect of 2,4-dichlorophenoxyacetic acid on photosynthesis and respiration. Plant Physiol. 29: 64–69.

PRODUCTS OF ORTHOPHOSPHATE ABSORPTION BY BARLEY ROOTS[1]

PATRICIA C. JACKSON[2] AND C. E. HAGEN[3]

AGRICULTURAL RESEARCH SERVICE, U. S. DEPARTMENT OF AGRICULTURE, BELTSVILLE, MARYLAND

The rate-limiting step of orthophosphate absorption by barley roots has been shown by kinetic studies (5) to be coupled to two of the sites of oxidative phosphorylation associated with isolated mitochondria. One of the reactions is coupled to reduced cytochrome b and the other to reduced diphosphopyridine nucleotide (DPNH). The present paper describes the identification of some of the products of the rate-limiting absorption step. The significance of the distribution of absorbed phosphate among these products and the rate of appearance in the products is discussed.

MATERIALS AND METHODS

The experimental plant material consisted of excised roots from 6-day old seedlings of barley (*Hordeum vulgare*, var. Atlas 46). The seedlings had been grown in the dark in continuously aerated 10^{-4} M $CaSO_4$, essentially as described by Epstein and Hagen (2). Roots were excised from the seedlings just prior to the absorption procedure, rinsed three times with 50 volumes of distilled-demineralized water, and suspended in 100 volumes of continuously aerated distilled-demineralized water.

Solutions for absorption experiments were single salt solutions of orthophosphate containing P^{32}, either in the absence or presence of various reagents under study. Sufficient volumes of the absorption solutions per mass of roots were used so that the concentration of phosphate and the reagents remained constant during the absorption. Hydrogen ion concentration was maintained to within 0.1 unit of pH. The excised roots were blotted with clean dry cheesecloth, weighed, and again rinsed three times with distilled-demineralized water before the absorption solutions were added. Absorption was terminated by rapid decantation of the solutions, followed by four rinses of the roots with 50 volumes of distilled-demineralized water.

The P^{32}-labelled components associated with the roots after absorption were extracted by grinding with four volumes of 95 % ethanol with a Pyrex mortar and pestle. The resulting root homogenate was centrifuged and aliquots of the supernatant fraction were taken for chromatography. Where concentration of the supernatant fraction was desirable, it was evaporated at room temperature under vacuum to approximately one-tenth the original volume.

Descending chromatograms were run at room temperature on 17 × 20 inch sheets of Whatman #1 or #3 filter paper. The filter paper had been washed by irrigation with a 20 : 50 : 30 parts, by volume, mixture of acetic acid, ethanol, and water, followed by irrigation with distilled-demineralized water, and dried before use. Both one- and two-dimensional chromatograms were run with various combinations of solvents. Seventeen different solvents were tried, of which the two listed below were the most satisfactory for separation of the P^{32}-labelled compounds in the root extracts.

I. *n*-butyl alcohol : proprionic acid : water—45 : 23 : 32 % by volume (1).

II. Iso-butyric acid : ammonium hydroxide : water —57 : 4 : 39 % by volume (9).

For identification of the P^{32}-labelled fractions, the extract was applied as a 1 cm wide band across one end of a sheet of Whatman #3 paper and chromatographed with one of the above solvents. The separated bands of P^{32}-labelled fractions were then located on the paper by means of radioautographs, cut out, and eluted with water. For separation of the fractions for kinetic studies of phosphate incorporation into the components, the extracts were applied to the chromatographic paper as 1 cm diameter spots. Radioactive spots on the chromatograms were located, cut out, and counted in an internal proportional counter to determine the amount of P^{32}.

Nucleotides were located on the chromatograms by visual observation under a mineralight ultraviolet lamp. Sugar compounds were located after spraying the chromatograms with aniline phthalate (10). Identification of the sugar compounds was aided by color reactions with the spray that were characteristic of the type of sugar. Compounds containing phosphorus were located by spraying with an ammonium molybdate (6) or an iron salicylate solution (12).

The eluted P^{32}-labelled components were further characterized by the following chemical and physical criteria: A. Ultraviolet spectra in acid, alkaline, and neutral solutions. B. Co-chromatography with a known compound in several solvents. C. Rates of hydrolysis in 0.1 *N* or 1 *N* HCl at 100° C. D. Stepwise decomposition of the components. E. Identification of the decomposition products. The known compound was carried through the same procedure as the root components for direct comparison.

Specific activities of the eluted P^{32}-labelled components were also determined. The P^{31} content was determined essentially by the method of Truog and Meyer (13) after the components were hydrolyzed.

RESULTS AND CONCLUSIONS

IDENTIFICATION OF ABSORPTION PRODUCTS. Extracting the roots with 95 % ethanol shows that 75 % of the total phosphate absorbed in 10 minutes is soluble in ethanol. Water or 10 % trichloroacetic acid

[1] Received July 24, 1959.

[2] Chemist, Mineral Nutrition Laboratory, ARS, USDA.

[3] Deceased.

(TCA) extract additional P^{32} from the ethanol insoluble fraction. However, a constant amount of activity is not extractable by water or TCA, irrespective of the absorption time. Thus, the activity associated with the insoluble fraction contains up to 16 % of the total absorbed P^{32} after 15 seconds absorption, but less than 2 % after 10 minutes absorption.

The ethanol soluble components were separated by paper chromatography. Chromatograms of the ethanol extracts show that P^{32}-labelled phosphate is incorporated into five major fractions (fig 1), which contain 95 % of the total P^{32} extracted by ethanol. The remaining 5 % of the ethanol soluble activity is distributed among at least ten components. The distribution of P^{32} among the five fractions is the same whether the ethanol extraction is carried out at 0° C or at room temperature. The additional activity extracted by water or TCA is identical to fraction IV of the ethanol soluble fractions. Thus, 80 to 95 % of the total absorbed phosphate appears in these five fractions. Although the extraction by water and TCA is somewhat more efficient, considerable hydrolysis of fractions I and III is associated with the use of these solvents. Two-dimensional chromatograms, using the same solvent in both directions, reveal that appreciable hydrolysis of some of the components occurs during chromatography with many of the strongly acidic irrigating mixtures. Thus, consideration was given to formation of artifactual components from hydrolysis of the fractions.

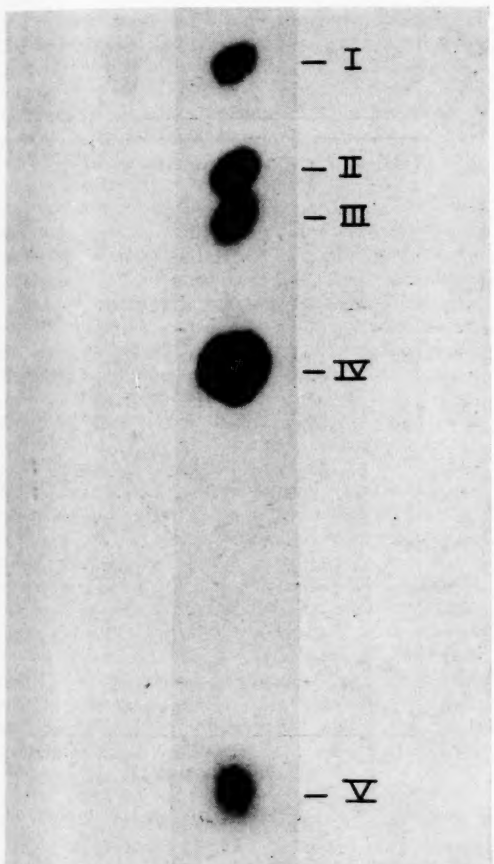


FIG. 1. Radioautograph of a chromatogram showing separation of P^{32} labeled components in excised barley roots. The solvent was a mixture of 57 % iso-butyric acid, 4 % ammonium hydroxide, and 39 % water.

The isolated P^{32}-labelled root fractions eluted from band chromatograms were chromatographed with some 40 phosphorus compounds commonly occurring in biological systems. Co-chromatography of a known compound with a root fraction is interpreted as only a tentative identification of the fraction since a pair of dissimilar compounds may co-chromatograph quite precisely in many different solvents. Failure of a fraction to co-chromatograph in any one solvent with a known compound is proof of dissimilarity of the compounds.

Fraction I (fig 1) co-chromatographs with uridine diphosphate glucose (UDPG) in all of the solvent mixtures tested. UDPG is identical with this fraction in ultraviolet absorption spectra in acid, alkaline, and neutral solutions, and in rates of hydrolysis. Products of partial decomposition of fraction I are the same as those of UDPG; namely, uridine diphosphate (UDP), uridine monophosphate (UMP), glucose, and inorganic phosphate. The terminal phosphate of the UDPG fraction contains 90 % of the total P^{32} of fraction I, with 10 % remaining in the phosphate of the UMP moiety.

Fraction III (fig 1) co-chromatograms with glucose-1-phosphate (G-1-P), mannose-6-phosphate, and galactose-1-phosphate. Separation of these compounds by other solvent systems was not attempted. However, the rate of hydrolysis of fraction III corresponds to that of G-1-P rather than that of mannose-6-phosphate or galactose-1-phosphate. Further, the formation of glucose upon decomposition rather than mannose or galactose indicates that most of fraction III is G-1-P.

Fraction IV (fig 1) was identified chemically (11) as well as chromatographically, as inorganic orthophosphate (P_i). Some of the P_i in fraction IV appears to be an artifact arising from hydrolysis of UDPG and, particularly, G-1-P during extraction of the roots and chromatography. The identity of fractions II and V remains unknown. Neither fraction co-chromatographed in all solvent mixtures with any of the known phosphate compounds, nor were the individual fractions further separable in any of the solvents. Only 10 % of either fraction hydrolyzes in 1 *N* HCl at 100° C in 4 hours.

No particular effort was made to identify any of the minor P^{32}-labelled components or unlabelled phosphate compounds in the roots. In the course of identification of the major components, however, a small fraction of P^{32} was found to be associated with uridine triphosphate. The root extracts contain some un-

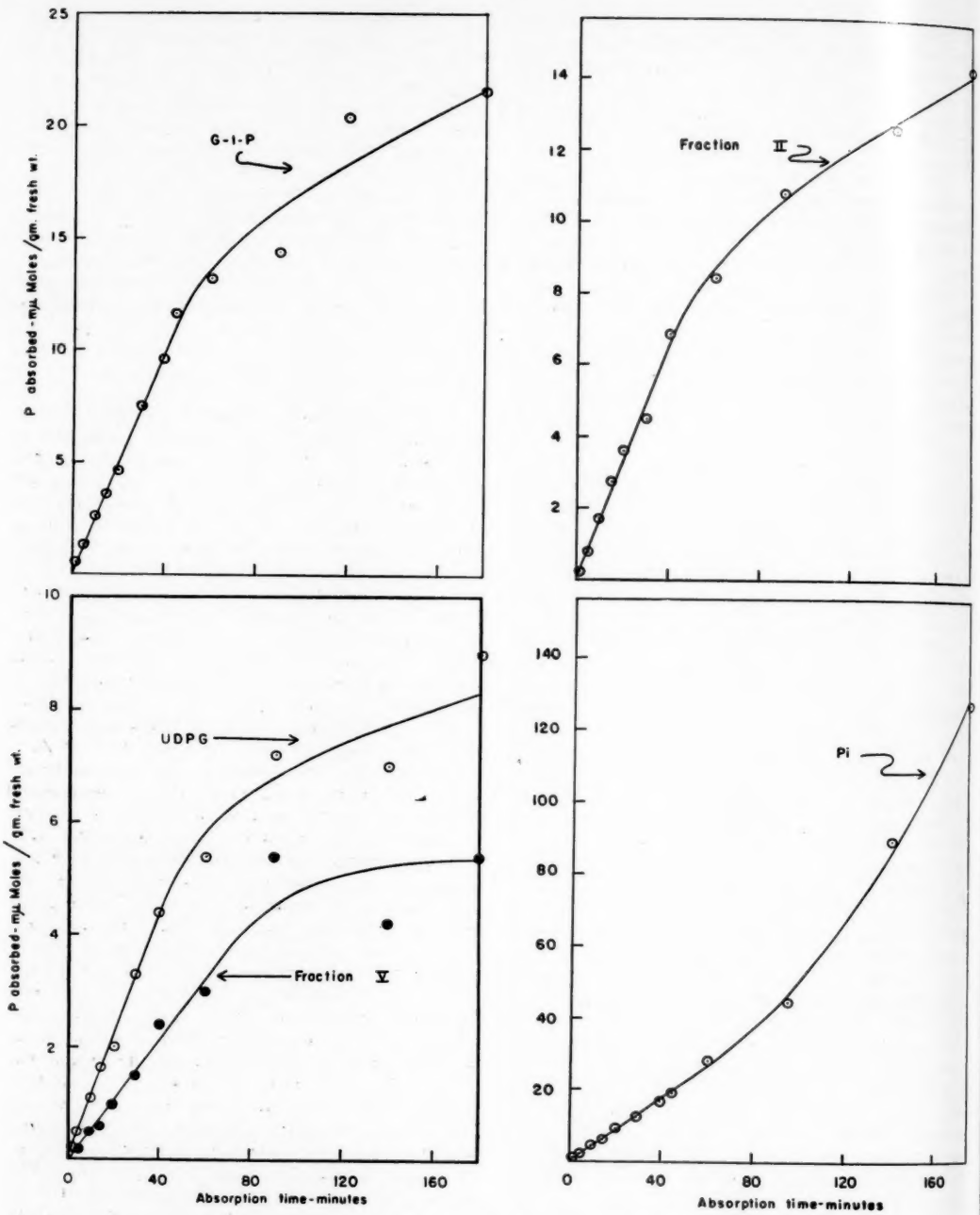


FIG. 2. Time course of P^{32} incorporation into the major phosphate absorption products in excised barley roots. Absorption was from 10^{-5} M phosphate at pH 4.

labelled adenosine diphosphate (ADP) and adenine, but no P^{32}-labelled adenosine phosphates. No measurable amounts of adenosine triphosphate (ATP) are present. In contrast to the roots, the leaves of barley plants contain relatively large amounts of adenosine monophosphate, ADP, and even greater amounts of ATP.

KINETICS OF P^{32} DISTRIBUTION IN ROOT COMPONENTS. The rate of phosphate absorption by the roots is constant for at least 4 hours. Figure 2 shows the time course of P^{32} incorporation into the five root fractions. The rate of the P^{32} appearance in UDPG and G-1-P is apparently constant for 40 minutes. Thereafter, the rate decreases. The incorporation rate into fraction II decreases after 50 minutes and, in fraction V, it decreases after 70 minutes. The time sequence of the rate decrease in these four fractions was always the same; i.e., UDPG and G-1-P, followed by fraction II, and last, fraction V. However, the rate of P^{32} appearance in P_i increased after 50 minutes of absorption with the rate increase compensating for the decreases in the rates in the other compounds. The long period of steady-state incorporation of P^{32} into the fractions excludes the possibility that any of them are initial sites or compounds formed prior to the rate-limiting absorption step. Thus, the five fractions must be products of phosphate absorption.

The decrease observed in the rate of P^{32} incorporation into four of the five fractions can be accounted for in two ways. One is that the amounts of the phosphate fractions increase significantly during phosphate absorption and the rate decreases when a mass equilibrium saturation is attained. In such a case, the time sequence of the rate decreases need have no relation to the sequence of formation of the fraction. The other possibility is that the fractions are in a dynamic equilibrium and do not increase significantly in amount during phosphate absorption. The appearance of P^{32} in the fractions, then, is a function of exchange of P^{31} for P^{32}. The fractions are in a sequence of reactions and the rate decrease occurs when the specific activity of the fractions approaches that of the incoming phosphate. The decrease in rate would occur in the same sequence of time as the sequence of incorporation.

To determine which of the two possibilities discussed occurs during phosphate absorption, the specific activity of the five separated fractions was determined after various absorption periods. Phosphate was absorbed by the roots from 10^{-5} M solution at pH 4 for intervals from 10 to 180 minutes. The amounts of the separated fractions were determined by chemical determination of phosphate and, in the case of UDPG, also by ultraviolet absorption at 260 mμ.

UDPG and G-1-P show no increase in total phosphate content in 180 minutes of absorption. The total phosphate of fractions II and V, and P_i increases by 10 to 20 %. In contrast, the specific activity of all fractions increases with time (table I). The rate of P^{32} incorporation into the five fractions is constant where the specific activity is below approximately

TABLE I

EFFECT OF ABSORPTION TIME ON SPECIFIC ACTIVITY OF P^{32}-LABELED ROOT FRACTIONS [1, 2]

ABSORPTION TIME MIN	P^{32} FRACTION				
	UDPG	II	G-1-P	P_i	V
10	2.7	3.6	6.9	0.54	1.5
30	8.4	10.3	13.5	1.33	2.4
60	12.7	14.8	16.5	2.0	4.8
90	27	23	53	4.7	7.0
180	33	27	58	11.4	14.4

[1] Roots had absorbed P^{32} from 10^{-5} M orthophosphate at pH 4.
[2] Percent of external phosphate specific activity.

15 % of the external phosphate specific activity. Thereafter, as the specific activity increases further, the incorporation rate decreases. In fraction V, the specific activity is only 14 % of the external phosphate specific activity after 180 minutes absorption and the rate is constant. The rate of P^{32} incorporation in P_i and its rate of specific activity increase increases with time.

It is apparent from this experiment that the break-off of the time curves of P^{32} appearance in G-1-P, UDPG and fractions II and V occurs because the specific activities of the fractions approach that of the incoming phosphate. The break-off appears to occur when the fraction specific activity exceeds 15 % of the external specific activity. Other experiments show that the time at which the break-off occurs is a function of the phosphate concentration and the specific activity of the absorption solution. The specific activity was always highest in UDPG, G-1-P and fraction II, lower in fraction V and lowest in P_i.

A study of the effect of P^{31} absorption on the distribution of P^{32} in the root fractions was carried out also to determine the sequence of the P^{32} incorporation into the five fractions. The roots had absorbed P^{32} for 60 minutes, a time at which equilibration of P^{32} among the fractions is just becoming apparent (fig 2). Under these conditions, the P^{31} absorbed would be expected to move P^{32} from the most primary components into later or final products. Roots which had absorbed P^{32} from a 10^{-5} M phosphate solution at pH 4 for 60 minutes were rinsed four times with distilled-demineralized water and placed in a 10^{-5} M solution of P^{31} phosphate at pH 4 for various intervals up to 2 hours. The same procedure was performed with phosphate solutions at pH 8, also. The amount of P^{32} absorbed was the same for all root samples at any one hydrogen ion concentration. Under these conditions, the roots continue to absorb P^{31} at the same rate as they have absorbed P^{32} without any loss of P^{32} from the roots. Results from the determination of the amount of P^{32} in each of the separated fractions (e.g., fig 3) show that the P^{32} is transferred from UDPG and G-1-P into fraction II and P_i. Fraction II initially gains P^{32} displaced from

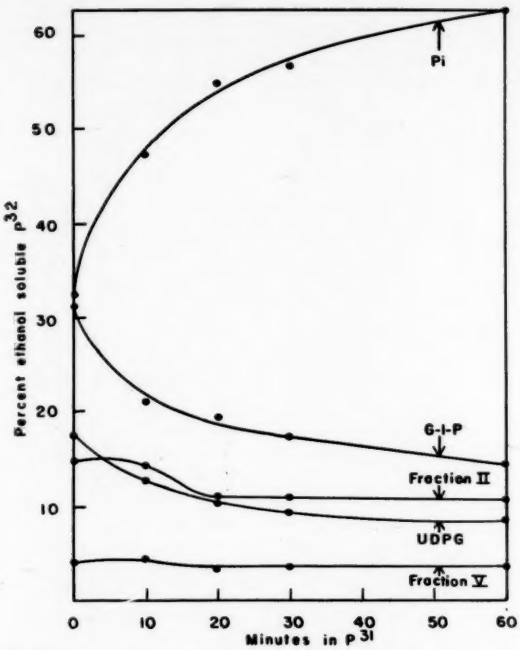
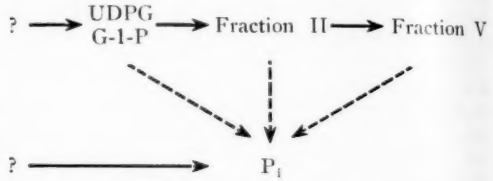


FIG. 3. Effect of P^{31} absorption on the P^{32} distribution in the root components. The roots had absorbed P^{32} for 60 minutes from a 10^{-5} M phosphate solution at pH 4, after which they were rinsed and placed in a 10^{-5} M P^{31} solution at pH 4.

one or both of the fractions, UDPG and G-1-P. Subsequently, activity in fraction II is also transferred to P_i. Continued incorporation of P^{31} has little or no effect on the P^{32} associated with fraction V, in this experiment. Fraction V eventually loses P^{32}, but in short times, little effect is apparent.

The influence of several sugars, namely, sucrose, fructose, glucose, mannose, galactose, and ribose, was studied to determine how much reactions subsequent to the rate-limiting absorption step alter the P^{32} distribution among the five fractions. Typical results are shown in table II. Previous experiments with C^{14}-labelled sucrose had shown that the sugar was absorbed and metabolized rapidly, with no effect on the accompanying phosphate absorption. None of the sugars tested in the present experiment alter the total amount of phosphate absorbed. The sugar concentration was 10^{-2} M in solutions of 10^{-6} or 5×10^{-4} M phosphate at pH 4. A pronounced shift in the distribution of P^{32} is produced by mannose, galactose, and ribose at either concentration of phosphate in the external solution. All of the sugars, except ribose, decrease the proportion of P^{32} in P_i with a proportionate increase of P^{32} in fraction II or G-1-P. Ribose decreases the P^{32} distribution in fraction V with a proportionate increase in fraction II. The P^{32} distribution in all of the five major fractions, except UDPG, is markedly affected by the sugars. The change in UDPG is relatively small and inconsistent. The sugars apparently form no new P^{32}-labelled fractions and only shift the distribution of P^{32} among the five major fractions. Thus, the effect of the sugars shows conclusively that the distribution of P^{32} among the five major fractions, with the possible exception of UDPG, is greatly influenced by reactions subsequent to the rate-limiting absorption step.

Experiments on the effect of P^{31} and the specific activity determinations show that the amount of P^{32} in the fractions is a function of P^{32}/P^{31} exchange. Therefore, a sequence of incorporation is inferred by the sequence of the breaks in the time curves. Thus:



All four fractions appear to contribute P^{32} to P_i. Incorporation into UDPG and G-1-P is so rapid that experiments do not show which of these two compounds precedes the other in sequence. There is a suggestion that UDPG may be the first by its relative insensitivity to the effect of sugars. Incorporation of P^{32} into fraction II is shown by the influence of P^{31} absorption (fig 3) to be subsequent to the incorporation into UDPG and G-1-P. That it is a product of UDPG and G-1-P rather than a precursor is suggested also by the effect of the sugars (table II), inasmuch as the P^{32} incorporation into this fraction can be increased without influencing incorporation into either UDPG or G-1-P. Fraction V is eliminated as a primary product by its relatively long period of steady-state incorporation, coupled with its low specific activity. The effect of ribose suggests that fraction V arises from fraction II. Fraction V does not

TABLE II

EFFECT OF SUGARS ON P^{32} DISTRIBUTION IN BARLEY ROOTS*

PERCENT OF ETHANOL SOLUBLE P^{32}

TREATMENT	P^{32} FRACTION				
	UDPG	II	G-1-P	P_i	V
Control	12	22	18	40	8
10^{-2} M glucose	15	27	20	30	8
10^{-2} M fructose	11	28	18	37	6
10^{-2} M mannose	8	6	70	12	4
10^{-2} M galactose	16	20	37	24	3
10^{-2} M ribose	13	30	19	38	1

* Roots had absorbed P^{32} from 10^{-6} M orthophosphate at pH 4 for 15 minutes.

appear to be an end product because eventually its rate of incorporation also decreases. Although some P^{32} in the P_i fraction comes from the other four fractions, short time experiments indicate that P^{32} appears in this fraction as soon as in UDPG and G-1-P.

Phosphate absorption occurs through two sites. The amount of phosphate absorbed through one of the sites, relative to the other is dependent upon the phosphate concentration and the pH of the external solution (4). Thus, if the products from one site differ from the other, the P^{32} distribution among the products should be in varying ratios dependent upon the limitations imposed by the absorption solution on the two individual sites. Further, the sequence of the P^{32} incorporation into the five major products suggests that UDPG, G-1-P and fractions II and V all are products of the same absorption site.

The influence of phosphate concentration and hydrogen ion concentration on the distribution of P^{32} among the root fractions was studied for comparison with the results of the kinetics of phosphate absorption. Short times of absorption were used to minimize the effects of equilibration among the products. Roots absorbed phosphate for 10 minute periods from solutions of 10^{-6} M to 5×10^{-4} M phosphate at pH 4 to 8. A decrease in the relative amounts of activity in UDPG, G-1-P and fractions II and V occurs with increasing phosphate concentration and increasing hydrogen ion concentrations (table III). According to the analysis of the kinetics of phosphate absorption (5), this decrease would be expected if all four of these fractions had arisen from the absorption coupled to cytochrome b. The proportion of P_i increases under these conditions, reflecting the absorption through the site that is apparently coupled to DPNH (5).

The effects of several inhibitors and activators on the distribution of P^{32} among the fractions were studied under conditions in which phosphate absorption coupled to cytochrome b would be affected. The inhibitors included pyrophosphate and nembutal, and the activators were calcium, succinate, and *p*-phenylenediamine. Representative results are shown in table III. Pyrophosphate and nembutal decrease the relative activity in UDPG, G-1-P and fraction II. Succinate, *p*-phenylenediamine and calcium increase the relative activities of these fractions, which again respond as if the absorption were coupled to cytochrome b. For example, the 30 % increase in total phosphate absorption induced by succinate, in table III, is entirely accounted for by the increase in the amount of P^{32} in UDPG, G-1-P and fraction II. The amount of P^{32} incorporated into P_i is unaffected. With all of the reagents, P_i responds as would absorption coupled to DPNH. The effects of the inhibitors and activators on fraction V were not as consistent with absorption through either site. This is not too surprising since fraction V is a later product than the others in sequence of P^{32} incorporation.

Some of the P^{32} incorporation into P_i by the roots arises from the release of phosphate from UDPG, G-1-P and fractions II and V, as shown by the relative changes in specific activity of the fractions with increasing absorption time (table I). This is also shown by the effect of P^{31} absorption on the distribution of P^{32} among the fractions (fig 3), and the decreased rates of P^{32} incorporation into UDPG, G-1-P and fractions II and V with a concomitant increase in the rate of incorporation into P_i (fig 2). However, not all of the activity in P_i arises from these compounds since P_i reflects the kinetics of absorption through the DPNH-linked site while UDPG, G-1-P and fraction II reflect the kinetics of the cytochrome b-linked site. These compounds qualitatively reflect the absorption kinetics but the shift in P^{32} distribution is not always as great as expected from the kinetics. This can be attributed in large measure to the transfer of P^{32} to P_i from the other four fractions. It is apparent, also, from the sugar experiments that the relative amounts of P^{32} incorporated into the fractions are greatly influenced by reactions subsequent to the rate-limiting absorption step.

TABLE III

EFFECTS OF VARIOUS AGENTS ON P^{32} DISTRIBUTION IN BARLEY ROOTS*

TREATMENT	% ETHANOL SOLUBLE P^{32}					TOTAL ABSORPTION
	P^{32} fraction					
10^{-6} *M Phosphate*	*UDPG*	*II*	*G-1-P*	*P_i*	*V*	*mμ M/min g fresh wt*
pH 4	13	21	21	38	7	1.0
pH 8	14	22	24	32	8	0.8
pH 4. 5×10^{-3} M calcium	15	24	24	34	3	1.2
pH 4. 10^{-3} M succinate	16	29	25	30	1	1.3
pH 4. 10^{-3} M nembutal	11	20	21	41	7	0.6
pH 4. 5×10^{-4} M pyrophosphate	8	11	7	68	6	0.5
5×10^{-4} *M Phosphate*						
pH 4	9	16	13	56	6	20.0
pH 8	13	19	14	46	8	12.0
pH 4. 5×10^{-3} M calcium	11	26	17	43	3	22.0
pH 4. 10^{-3} M nembutal	7	8	7	74	3	15.0

* Roots had absorbed P^{32} for 10 minutes.

Discussion

Miettinen and Savioja (8) found that roots of 6-week old pea plants grown in a nutrient solution incorporated P^{32} into ATP, UTP, and GTP in 10 seconds. Loughman and Russell (7) consider five nucleotides as the earliest products of 5 minute P^{32} absorption by young barley plants. They identified ATP and ADP as two of the nucleotides, but the others remain unknown. In the present experiments with etiolated barley roots, the most primary products of phosphate absorption among five major components which incorporate P^{32} within 15 seconds are UDPG, G-1-P and P_i. These results present no particular disagreement with those of Miettinen and Savioja or Loughman and Russell in as much as experimental factors differ greatly. UDPG was found by Ginsburg, Stumpf, and Hassid (3) to be one of the major nucleotide components of 5-day old mung bean seedlings. The amount of UDPG they found calculates to be approximately 5×10^{-8} M/g fresh weight. By comparison, barley roots in our experiments contain 7×10^{-8} M/g fresh weight. Ginsburg et al also found an approximately equal amount of AMP but only a fourth as much ADP as UDPG. This agrees with the relatively low level of adenosine compounds found in the barley roots.

Phosphate absorption by barley roots is coupled to oxidative phosphorylation. Since ATP has been shown to be the primary product of oxidative phosphorylation, its presence was expected to be among the P^{32}-labelled components of the root extracts. Yet, no P^{32}-labelled ATP was found. The explanation of this lies in consideration of the absorption kinetics. Analysis of the kinetics of phosphate absorption indicates that the absorption rate is not limited by the rate of ATP turnover; that ATP turnover must be as fast or faster than the rate-limiting absorption step. As a result, the concentration of ATP would be maintained at a low level.

The large percentage of the absorbed P^{32} appearing as inorganic orthophosphate is derived as a product of internal metabolism and breakdown of a labile compound. The fact that the P_i fraction is not exchangeable with phosphate in the external absorption solution and is apparently a product of DPNH-linked absorption suggests that it must have been in some high energy combination. Teleologically, the formation of only P_i from a high energy phosphate combination seems inconsistent unless energy is conserved through formation of a labile compound such as acetyl phosphate. It is apparent from the present studies, however, that the turnover rates for the individual products of phosphate absorption are so rapid that the initial precursor can not be identified by observation of the labelling of the phosphate esters.

Summary

Orthophosphate absorbed by barley roots is incorporated into five major compounds which incorporate 80 to 90 % of the total phosphate absorbed in periods from 15 seconds to 2 hours. The kinetics of the incorporation into each of the compounds shows that all five are products of the rate-limiting absorption step. UDPG, G-1-P and P_i appear to be the earliest compounds among the five in sequence of phosphate incorporation. Four of the compounds, UDPG, G-1-P and two unknowns arise from absorption through one site and P_i arises from another. This is shown by the sequence of P^{32} incorporation and the differential effects of phosphate concentration, pH and various inhibitors and activators. Further, the specific effects of the activators and inhibitors suggest that UDPG, G-1-P and the two unknowns arise from absorption coupled to cytochrome b of the respiratory chain. P_i is apparently a product of DPNH-linked absorption.

Acknowledgements

The authors wish to express gratitude to Dr. S. B. Hendricks and Mr. J. E. Leggett for their interest, criticism, and assistance.

Literature Cited

1. Benson, A. A. 1957. Sugar phosphates, paper, and column chromatography. In: Methods in Enzymology III, S. Colowick, ed. 110–129.
2. Epstein, E. and C. E. Hagen 1952. A kinetic study of the absorption of alkali cations by barley roots. Plant Physiol. 27: 457–474.
3. Ginsburg, V., P. K. Stumpf, and W. Z. Hassid 1956. The isolation of uridine diphosphate derivatives of D-glucose, D-galactose, D-xylose and L-arabinose from mung bean seedlings. Jour. Biol. Chem. 233: 977–983.
4. Hagen, C. E. and H. T. Hopkins 1955. Ionic species of orthophosphate absorption by barley roots. Plant Physiol. 30: 193–199.
5. Hagen, C. E., J. E. Leggett, and P. C. Jackson 1957. The sites of orthophosphate uptake by barley roots. Proc. Nat. Acad. Sci. 43: 496–506.
6. Hanes, C. S. and F. A. Isherwood 1949. Separation of the phosphoric esters on the filter paper chromatogram. Nature 164: 1107.
7. Loughman, B. C. and R. S. Russell 1957. The absorption and utilization of phosphate by young barley plants. IV. The initial stages of phosphate metabolism in roots. Jour. Exp. Bot. 8: 280.
8. Miettinen, J. K. and T. Savioja 1958. Uptake of orthophosphate by normal and P-deficient pea plants. Suomen Kemistilehti 31: 84–90.
9. Pabst Laboratories. 1956. Ultraviolet absorption of 5′ ribonucleotides. Circular OR-10, p. 20.
10. Partridge, S. M. 1949. Aniline hydrogen phthalate as a spraying reagent for chromatography of sugars. Nature 164: 443.
11. Pons, W. A. and J. D. Guthrie 1946. Determination of inorganic phosphorus in plant materials. Ind. Eng. Chem., Anal. Ed. 18: 184–186.
12. Runeckles, V. C. and Gleb Krotkov 1957. The separation of phosphate esters and other metabolites by ionophoresis and chromatography on paper. Arch. Biochem. Biophys. 70: 442–453.
13. Truog, E. and A. H. Meyer 1929. Improvements in the Deniges colorimetric method for phosphorus and arsenic. Ind. Eng. Chem., Anal. Ed. 1: 136–139.

PROMOTION OF LETTUCE SEED GERMINATION BY GIBBERELLIN[1,2]

ALBERT KAHN

BOTANY DEPARTMENT, UNIVERSITY OF CALIFORNIA, LOS ANGELES

Lettuce seeds do not germinate in darkness under certain conditions, and their germination can then be promoted by many factors. Kahn et al (9, 10) have presented preliminary reports on the promotion of dark germination of Grand Rapids lettuce seeds by gibberellin. Some details of their work and more recent results of the study will be given here. Some of the previously reported findings have been confirmed and amplified by researchers at The Hebrew University of Jerusalem (7, 15). Also, Lona (14) has found that gibberellic acid promotes dark germination of a wild lettuce species, *Lactuca scariola* L.

Among the factors which may inhibit dark germination of lettuce seeds are: supra-optimal germination temperature (3, 5); pretreatment of imbibed seeds with high temperature (1, 2); and a process called "dark-osmotic inhibition" (8). Dark-osmotic inhibition is the reduction of germination in darkness by the incorporation of appropriate amounts of osmotically active solute in the germination solution. The above inhibitory factors may all induce the same ultimate block in the germination pathway. Often lettuce seeds whose germination is inhibited by one of these factors will germinate if they are irradiated briefly with red light. Such seeds may be termed "red-light responsive". When a red irradiation is followed by a far red irradiation, the action of red light is reversed (1, 2). Following the far red irradiation, the promotion of germination can again be potentiated by a red irradiation, and such a promotion and reversal can be accomplished many times.

The effect of gibberellin upon germination was determined on seeds that were made red-light responsive by supra-optimal germination temperature, dark-osmotic inhibition, pretreatment with high temperature, or irradiation with red light followed by irradiation with far red light.

Gibberellin causes bolting and flowering of long day plants in short days (12, 13). Because the light action spectra for the control of flowering and seed germination are similar (2), a part of this investigation was devoted to examining the relationship of gibberellin and light actions in seed germination.

[1] Received July 31, 1959.

[2] This work was supported in part by a grant made by the Lilly Research Laboratories to Professor Anton Lang and in part by a research grant (RG–3939) to Professors Lang and Sam G. Wildman from the National Institutes of Health, U.S. Public Health Service.

MATERIALS AND METHODS

SEEDS: Three lots of Grand Rapids lettuce seed (*Lactuca sativa* L.) were used in this study. Their approximate germination range at 21° C in darkness on water-saturated filter paper during this work and their sources are given in table I. Lot No. 1 is the same as that used previously to study dark-osmotic inhibition (8).

GERMINATION CONDITIONS AND LIGHT SOURCES: The standard experimental procedure has been described in detail previously (8). All modifications of this procedure will be noted. This, briefly, is the general method employed. Each replicate of a treatment consisted of 100 seeds sown in a Petri dish on Whatman no. 1 filter paper saturated with an experimental solution. Unless a contrary statement is made, from the time the seeds were sown the dishes were kept continuously in moist chambers in darkness at $21 \pm 1°$ C. All operations requiring vision were performed in dim green light. Red light was obtained from cool white fluorescent tubes and far red light from reflector photoflood lamps, with appropriate filters.

CHEMICALS: C.P. grade mannitol was used. Two gibberellin preparations were used: A. A mixture ($[a]_D^{25} + 62°$) of gibberellin A_1 (gibberellin A) and gibberellin A_3 (gibberellic acid); and B. A sample of gibberellin A_3, 88.9 % pure. The generic term gibberellin will be used for both preparations; in the description of experiments the second preparation will be distinguished from the first by calling the second gibberellic acid.

TABLE I

DESCRIPTION OF GRAND RAPIDS LETTUCE SEED LOTS USED IN THIS STUDY

SEED LOT DESIGNATION	% GERMINATION*	SOURCE
1	70–95	Ferry - Morse Seed Co., Mountain View, Cal., 1953
2	20–35	Dr. Carlos O. Miller, Dept. of Botany, University of Wisc., Madison; now Dept. of Botany, Ind. University, Bloomington, 1956
3	45–70	Ferry - Morse, Tip - Burn Resistant strain, 1957

* On water-saturated filter paper at 21° C in darkness

TABLE II

PROMOTION OF GERMINATION OF SEED LOT NO. 2 BY RED LIGHT AND BY GIBBERELLIN

RED LIGHT (MIN)*	GIBBERELLIN (MG/L)	% GERMINATION	
		REPLICATES	AVERAGE
0	0	27, 20	24
2	0	73, 72	73
3	0	72, 67	70
0	100	71, 69	70
0	100**	75, 72	74
0	200	70, 69	70

* Administered 2 hours after the beginning of imbibition

** Gibberellic acid

RESULTS

RELEASE BY GIBBERELLIN OF INHIBITION CAUSED BY SUPRA-OPTIMAL GERMINATION TEMPERATURE: For different lots of Grand Rapids lettuce seeds various temperatures can be found which are supra-optimal for germination in darkness (5, unpublished data). For lot No. 2, 21° C was too high to permit more than 20 to 35 % germination on water in darkness, but a brief irradiation with red light strongly promoted germination at that temperature. The promotive effect of gibberellin and of red light on the germination of this seed lot is shown in table II. This seed lot was peculiar in that maximal germination was never obtained with a germination period of 48 or fewer hours or with a single red irradiation. Therefore, the saturation of the effects of light and gibberellin that are evident in the table do not mean that about 25 % of this seed lot was non-viable or insensitive to light and gibberellin. Almost all of the non-germinating seeds from experiments with this lot germinated within several days when left in their dishes on a laboratory bench.

EFFECT OF GIBBERELLIN ON DARK-OSMOTIC INHIBITION: The nature of dark-osmotic inhibition has been analyzed previously (8). Seeds sown on a solution containing sufficient solute to prevent germination in darkness, germinate fully if irradiated briefly with red light. When gibberellin (35 mg/l) is included in a germination solution of 0.15 M mannitol, the effectiveness of dark-osmotic inhibition is reduced. This response is shown in table III. Since red light protects the seeds against dark-osmotic inhibition (8), it seemed worthwhile to determine whether or not gibberellin pretreatment also can reduce the effectiveness of dark-osmotic inhibition. Seeds may imbibe water for several hours, and their germination can still be inhibited by subsequent transfer to an osmotic solution (8). Accordingly, seeds allowed to imbibe gibberellin (50 mg/l) or water for 6 hours were washed thoroughly with water, and transferred to 0.15 M mannitol. Table IV shows the promotive effect of gibberellin upon the germination of seeds on 0.15 M mannitol when gibberellin is supplied during a pretreatment. Although the external gibberellin supply was withdrawn from the seeds after 6 hours of uptake, the pretreatment with gibberellin nullified dark-osmotic inhibition of germination. Gibberellin did not necessarily act during the pretreatment, as gibberellin that probably remained within the seeds, despite washing, could have acted later.

TABLE III

EFFECT OF GIBBERELLIN ON DARK-OSMOTIC INHIBITION*

MANNITOL (MOLARITY)	GIBBERELLIN (MG/L)	% GERMINATION	
		REPLICATES	AVERAGE
0	0	76, 87	82
0.15	0	25, 18	22
0.15	35	65, 56	61

* Seed lot No. 1

EFFECT OF GIBBERELLIN ON INHIBITION OF GERMINATION CAUSED BY HIGH TEMPERATURE PRETREATMENT: Borthwick et al (1, 2) have shown that lettuce seeds of some varieties germinate well in darkness at a particular temperature (20-25° C), but do not germinate well at that temperature if transferred to it after imbibing water and being maintained at 30 to 35° C for one to four days. This treatment will be called "heat sensitization", and seeds that are dark-dormant at 21° C because they have had such a treatment will be referred to as "heat sensitized". The germination of such seeds can be promoted by red light.

TABLE IV

EFFECT OF GIBBERELLIN PRETREATMENT ON DARK-OSMOTIC INHIBITION

PRETREATMENT (6 HRS)	% GERMINATION*	
	REPLICATES	AVERAGE
Water	23, 23	23
Gibberellin (50 mg/l)	86, 88	87

* Seed lot No. 1 germinated on 0.15 M mannitol after transfer from water or gibberellin

To study the effect of gibberellin on heat sensitization, seeds of lot No. 1 were allowed to imbibe either water or a solution of gibberellin (50 mg/l) while kept at 36 ± 1° C for 117 hours. Germination was less than 1 % during this period. After this time all seeds were allowed to germinate at 21° C for 49.5 hours. Table V gives the results of this experiment. When gibberellin is present during a pretreatment with high temperature no effect of heat sensitization becomes apparent. This finding can be explained in two ways: A. seeds do not become heat sensitized in the presence of gibberellin; B. gibberellin permits seeds to circumvent that block to germination which results from heat sensitization.

TABLE V

GERMINATION* AT 21° C FOLLOWING HIGH TEMPERATURE PRETREATMENT** IN PRESENCE OR ABSENCE OF GIBBERELLIN

GIBBERELLIN (50 MG/L)	% GERMINATION	
	REPLICATES	AVERAGE
...	12, 26, 28, 16, 20	20
+	91, 96, 90	92

* Seed lot No. 1
** 36 ± 1° C for 117 hours

The following experiment was performed to determine if gibberellin can promote the germination of seeds that had become heat sensitized. Seeds of lot No. 1 were sown on water-saturated filter paper and stored at 36±1° C for 93.5 hours. They were then dried in darkness at 36° C for 23.5 hours without removal from the dishes. After drying of the seeds and the filter paper substratum, sufficient amounts of water or of gibberellin solution were added to each dish to saturate the filter paper. All seeds were then kept at 21° C for 49.5 hours before germination counts were made. Table VI shows that gibberellin treatment following heat sensitization promotes germination. Thus, gibberellin enables the seeds to overcome or circumvent the germination block that results from heat sensitization. One cannot test to learn if gibberellin can also prevent heat sensitization, since gibberellin that is supplied during the high temperature treatment cannot be removed from the seeds. It may cause germination after the seeds are shifted to a lower temperature.

PROMOTION OF GERMINATION OF SEEDS THAT HAD BEEN IRRADIATED WITH RED LIGHT FOLLOWED BY FAR RED LIGHT: Promotion of lettuce seed germination is frequently potentiated by a brief red irradiation, and this effect is reversed by a brief far red irradiation (1, 2). Seeds that have received sufficient red light to potentiate germination and soon thereafter sufficient far red light to reverse the potential to germinate will be called "far red inhibited", although far red light may do no more than nullify the action of red light and restore the seeds to their original condition. Red light can repotentiate the germination of far red inhibited seeds (1, 2). The following two experiments were performed to find out whether gibberellin also promotes the germination of far red inhibited seeds. Two alternatives are given for the length of high temperature pretreatment, temperature during drying of the seeds, gibberellin concentration, and length of the germination period. In each case, the first of the alternatives refers to conditions of experiment 7, and the second to conditions of experiment 8 (table VII). Seeds of lot No. 3 were sown on water-saturated filter paper and kept at 33±2° C for 28 2/3 or 31 hours. At the end of these periods, some seeds received 30 minutes of red light; some 30 minutes of red light followed by 1 minute

TABLE VI

PROMOTION OF GERMINATION AT 21° C OF HEAT SENSITIZED SEEDS* BY GIBBERELLIN

REIMBIBED SOLUTION	% GERMINATION	
	REPLICATES	AVERAGE
Water	40, 12, 26	26
Gibberellin (50 mg/l)	58, 47, 31	45
Gibberellin (100 mg/l)	81, 55, 68	68

* Seeds of lot No. 1 were sown on water and kept at 36 ± 1° C for 93.5 hours. They were then air-dried at 36° C and allowed to reimbibe on experimental solutions.

TABLE VII

PROMOTION OF GERMINATION OF FAR RED INHIBITED SEEDS* BY GIBBERELLIC ACID

REIMBIBED SOLUTION	IRRADIATION DURING PRETREATMENT		% GERMINATION		
	RED LIGHT (30 MIN)	FAR RED LIGHT (1 MIN)	REPLICATES EXPT. 7	REPLICATES EXPT. 8	AVERAGE
Water	...	...	36, 43, 52, 56		47
Water	+	...	82, 71, 79, 74	74, 78, 74, 80, 73, 75	76
Water	+	+	15, 17, 31, 20	9, 5, 7, 10, 6, 6	13
Gibberellic acid (1,000 or 1,500 mg/l)	...	...	89, 88, 85, 77		85
Gibberellic acid (1,000 or 1,500 mg/l)	+	+	72, 70, 78, 81	84, 72, 83, 79, 72, 85	78

* Seeds of lot No. 3 were partially heat sensitized, irradiated as shown in the table, air dried, allowed to reimbibe on the solutions shown in the table, and germinated at 21° C.

of far red light; and some received no irradiation. The seeds were then air dried in darkness at high temperature or at 21° C. No seeds germinated during these pretreatments. Subsequently, sufficient water or gibberellic acid (1,000 or 1,500 mg/l) to saturate the filter papers was added to each dish, and the seeds were then allowed to germinate at 21° C for 74 or 51 hours before germination counts were made.

Table VII shows that the far red irradiation following the red irradiation reduced germinability of the seeds on water from 76 to 13 %. The germination of seeds pretreated with red followed by far red light was promoted to 78 % by treatment with gibberellic acid. Therefore, gibberellic acid promotes the germination of far red inhibited seeds. High gibberellic acid concentrations were used, because in earlier experiments with the same seed lot, 400 mg/l elicited only a small promotion of germination of seeds receiving similar, but longer high temperature pretreatment and either no irradiation or red followed by far red irradiations.

ARE MECHANISMS OF ACTION OF LIGHT AND GIBBERELLIN RELATED? As shown above, gibberellin can substitute for red light in promoting the germination of lettuce seeds that do not germinate in darkness because of relatively low supra-optimal germination temperature, dark-osmotic inhibition, heat sensitization, or far red inhibition. The identical end effect of two factors, however, can never be taken as evidence for identical mechanisms of action. The following experiments were performed to determine whether or not there is some kind of relationship between the mechanisms of action of light and gibberellin in lettuce seed germination.

Since the primary action of red light can be fully reversed by a subsequent, brief far red irradiance (1, 2, 8), an attempt was made to find out if promotion of germination by gibberellin could be negated by far red light. Seeds of lot No. 2 were sown on water or gibberellin (10 mg/l). The promotion of germination by this gibberellin concentration was approximately the lowest that could be detected with assurance. Half the dishes of seeds on each substratum were irradiated for 2 minutes with far red light on three occasions: 4, 19½, and 25¾ hours after the beginning of imbibition. At the intensity used, two minutes of far red light were always sufficient to reverse fully the action of a saturating dose of red light given immediately before the far red irradiation. The total germination period was 49.5 hours. The first group of results in table VIII shows that the promotion of germination by gibberellin was not reduced by three brief irradiations with far red light.

Two similar experiments done with seed lot No. 1 are summarized by the second group of data in table VIII. Seeds were sown on 0.22 M mannitol with or without gibberellin (100 mg/l) incorporated in the solutions. Seeds were irradiated three or four hours after they were sown. The total germination periods were 50 or 53.5 hours. Twenty seconds of far red light were ample to reverse fully the effect of a prior red irradiation. Reduction of germination by far red light of seeds supplied with gibberellin was somewhat greater than that of seeds lacking gibberellin. Thus, far red light may have reduced gibberellin action slightly in this case, but as the differences are small, this conclusion rests on an infirm basis and is inconsistent with the first group of data in the table.

Though in these experiments brief irradiances of far red light did not negate promotion of germination by gibberellin, it is still conceivable (especially supposing that gibberellin is not metabolized by the seeds) that far red light does reverse gibberellin action. One might assume that any effect produced by gibberellin prior to a brief far red irradiation is in fact reversed by the irradiation, but that residual gibberellin in the seed immediately acts again to promote germination. Therefore, experiments were performed in which far red light was given continuously. Seeds of lot No. 3 were used and temperatures (26 or 27±1° C) that were supra-optimal for dark germina-

TABLE VIII

STABILITY OF GIBBERELLIN EFFECT TO BRIEF IRRADIANCES OF FAR RED LIGHT

MANNITOL (0.22M)	GIBBERELLIN (MG/L)	RED LIGHT (5 MIN)	FAR RED LIGHT	% GERMINATION		
				REPLICATES		AVERAGE
				Expt. 6*		
	0	...	...	18, 21, 18, 20, 27		21
...	0	...	3 × 2 min	29, 22, 23, 27, 21		24
	10	...	...	41, 35, 38, 39, 25		36
	10	...	3 × 2 min	33, 43, 46, 29, 30		36
				Expt. 50**	Expt. 54**	
	0	...	...	14, 16	28, 21, 23	20
+	0	...	20 sec	18, 6	14, 15, 14	13
	100	...	...	25, 35	49, 48, 40	39
	100	...	20 sec	19, 36	16, 32, 26	26
	0	+	...	80, 86	97, 93, 92	90
	0	+	20 sec	21, 23	20, 17, 9	18

* Seed lot No. 2
** Seed lot No. 1

TABLE IX

INHIBITION OF GERMINATION* BY CONTINUOUS FAR RED LIGHT

SOLUTION	FAR RED LIGHT	% GERMINATION		AVERAGE
		REPLICATES		
		EXPT. 19	EXPT. 20	
Water (control)	...		5, 2, 0, 1	2
Water (control)	+		0, 0, 0, 0	0
Water**	...	78, 79, 76, 68		75
Water**	+	5, 4, 3, 3		4
Gibberellic acid (400 or 200 mg/l)	...	41, 81, 73, 55	67, 62, 62, 78	65
Gibberellic acid (400 or 200 mg/l)	+	6, 3, 2, 6	10, 2, 6, 3	5
Thiourea (0.5 or 0.2 %)	...	29, 48, 50, 54	58, 33, 42, 36	44
Thiourea (0.5 or 0.2 %)	+	13, 6, 2, 2	2, 1, 0, 1	3

* Seed lot No. 3 at 26 or 27±1° C with 72 or 70 hours allowed for germination
** Pretreated at 4±1° C in darkness for 8 days

tion of this lot were employed. Two factors that promote dark germination of lettuce seeds at supra-optimal temperature were compared with gibberellic acid. They were low-temperature pretreatment (3) and thiourea (16). The results of two such experiments are summarized in table IX. Seeds receiving a cold pretreatment were kept imbibed in darkness at 4±1° C for 8 days. Thiourea was used as a 0.5 or 0.2 % solution, gibberellin was used at a concentration of 400 or 200 mg/l, and germination counts were made after 72 or 70 hours. It is evident that the low temperature pretreatment, gibberellic acid, and thiourea all promoted germination in darkness but not in continuous far red light.

In experiment 19 (table IX), seeds which after 72 hours on 400 mg/l gibberellic acid in far red light had germinated 4 %, were tested further. The counting and transfer to darkness were done by the light of the far red source. Two of the four replicates were left in continuous far red light, and two replicates were shifted to darkness, where they remained for an additional 48 hours. At the end of this period, seeds remaining in far red light had germinated 68 % and those shifted to darkness had germinated 58 %. This 10 % difference is not considered significant. Thus, far red light under conditions of these experiments does not prevent gibberellin-promoted germination, but it does delay it markedly. However, the delaying action of far red light on gibberellin-promotion of germination may not be specific, since continuous far red light also prevents or delays the promotion of germination by other factors that enhance lettuce seed germination in darkness.

Another approach to the question of whether a relationship exists between the mechanisms of action of red light and gibberellin was a study of the combined effects of these two treatments. As a rule, if the effects of two different factors upon a given response are more than additive, a synergistic interaction of the factors is indicated. Per cent germination being an expression of ultimate all-or-none responses by individuals comprising a sample of a population, however, attempts at deciding whether the *actions* of gibberellin and red light are additive or synergistic, based on the percentage responses of the seeds, must be considered tentative.

The combined effects of red light and gibberellin upon germination have been studied in many experiments. Whenever feasible, data from such experiments have been grouped for presentation in table X. Experiments that were united always yielded the same conclusions individually. All groups of data representing 6 or more replicates (each of 100 seeds) per treatment are based upon the averaged results of two or three separate experiments. Since experiments were not always repeated in precisely the same manner, in some cases alternative mannitol or gibberellin concentrations or irradiances of red light are given in the grouped data. The table shows that the combined effects of gibberellin and red light are additive or somewhat less than additive, if the irradiation is given four or fewer hours after the seeds are sown. When the irradiation is given 20 or more hours after the seeds are planted, however, the combined effects of red light and gibberellin are somewhat greater than additive.

This apparent synergistic effect is relatively small but quite consistent. It is most evident in the last experiment in table X. Here a 5 second red irradiance given 28 hours after the beginning of imbibition did not produce a measurable effect by itself, but, when combined with 50 or 100 mg/l of gibberellic acid, the red irradiance increased germination from 16 to 35 and from 36 to 58 %, respectively. In other words, presence of gibberellic acid seems to have resulted in an increased responsiveness of seeds to red light.

DISCUSSION

A close parallel has been established between the effects of gibberellin and red light on lettuce seed ger-

mination. Both promote dark germination of seeds which would not germinate because of relatively low supra-optimal germination temperature, heat sensitization, dark-osmotic inhibition, or far red inhibition.

Brief irradiation of seeds with far red light does not negate gibberellin action (see table VIII). Perhaps promotion of germination by gibberellin is reduced slightly by a brief far red irradiance, but such an effect is not shown consistently. Evenari et al (7) experienced the same results (see their tables IV and V). Because of this lack of consistency, and because of the difficulty in interpreting ineffectiveness of brief far red irradiations as noted previously, the combined data at hand do not permit us to decide whether or not brief far red irradiances can reverse any part of the promotion caused by gibberellin. Therefore, these data do not allow a conclusion about the relation of the effects of light and gibberellin in lettuce seed germination.

The effect of prolonged irradiations with far red light also permit no conclusion about the relation of light and gibberellin in lettuce seed germination. Clearly, continuous far red light of rather high intensity delays the germination of seeds promoted by gibberellin. This need not indicate specific reversibility of gibberellin action by far red light comparable with the reversal of the action of red light by subsequent brief far red radiation, since the germination of seeds promoted by thiourea or a low temperature pretreatment is also delayed or prevented by continuous far red light.

Experiments in which gibberellin and red light were given in combination showed that their effects are simply additive when seeds are irradiated early in the germination period, but are more than additive when a red irradiance is given 20 or 28 hours after the beginning of imbibition. Data of Poljakoff-Mayber et al (15; see their table IV) also show more than additive effects when lettuce seeds are irradiated with red light 48 hours after the beginning of imbibition on gibberellin solution. Consequently, gibberellin not only promotes lettuce seed germination in complete darkness, but also, after a sufficiently long period of action, it seems to increase the responsiveness of lettuce seeds to red light. It is known that the responsiveness of imbibing lettuce seeds to red light under-

TABLE X

PROMOTION OF GERMINATION BY COMBINATIONS OF GIBBERELLIN AND RED LIGHT TREATMENTS

SEED LOT NO.	NO. REPLICATES/ TREATMENT	MANNITOL (MOLARITY)	GIBBERELLIC ACID (MG/L)	RED LIGHT (SEC)	TIME OF RED LIGHT APPLICATION*	% GERMINATION (AVG)	INCREASE OVER CONTROLS	†
1	9	0.22	0	0	3 or 4	23	...	
			100	0		48	25	
			0	40		48	25	
			100	40		72	49	50
2	10	0	0	0	4	21	...	
			10**	0		38	17	
			0	10		35	14	
			10**	10		47	26	31
3	8	0.10	0	0	4	11	...	
			20 or 25	0		47	36	
			0	10		21	10	
			20 or 25	10		56	45	46
3	4	0.15	0	0	4	0	...	
			50	0		16	16	
			0	10		0	0	
			50	10		16	16	16
3	8	0.10 or 0.15	0	0	16	6	...	
			10 or 50	0		23	17	
			0	4 or 10		21	15	
			10 or 50	4 or 10		45	39	32
3	6 or 8	0.15	0	0	20	1	...	
			30 or 50	0		14	13	
			0	10 or 6		26	25	
			30 or 50	10 or 6		55	54	38
3	4	0.15	0	0	28	1	...	
			50	0		16	15	
			100	0		36	35	
			0	5		3	2	
			50	5		35	34	17
			100	5		58	57	37

* Hours from beginning of imbibition to irradiation
** Mixture of gibberellin A and gibberellic acid
† Increase over controls expected for simple addition of effects of red light and gibberellin alone

goes a rapid initial rise, plateaus, and then declines rapidly after 12 to 20 hours of water uptake by the seeds (2, 6). Since synergism between gibberellin and red light action, as measured by per cent germination, becomes substantial about 20 hours after sowing of the seeds, it seems possible that a function of gibberellin is to maintain the responsiveness of imbibed seeds to red light at the maximum level for a longer period. An alternative notion is that gibberellin must be transformed or affect a preparative process before it influences responsiveness of seeds to red light, and about 20 hours are required for the gibberellin action to become effective.

These results lead to the suggestion that gibberellin may affect seed germination along two different pathways: a. Along a red light dependent pathway, and b. Along an alternate pathway that functions in darkness and is not necessarily related to the former. Possibly these two pathways are developed in varying degrees in diverse species of seeds.

In lettuce seeds and in seeds of *Arabidopsis thaliana* (L.) Heynh. (11), where gibberellin also is able to replace red light in germination, i.e., to induce germination in total darkness, the major part of gibberellin action seems to be along the pathway which is not dependent on light. On the other hand, in seeds of *Kalanchoë blossfeldiana* v. Poellnitz, where gibberellin greatly reduces the daily light requirement for germination but at a concentration of 100 mg/l fails to bring about any dark germination (4), the major or entire gibberellin action may be to alter the light-dependent pathway.

Summary

I. Gibberellin causes dark germination of lettuce seeds that would not germinate in the absence of a promoter because of a relatively low supra-optimal germination temperature; dark-osmotic inhibition: inhibition induced by a high temperature pretreatment, and inhibition produced by far red light.

II. Thus, gibberellin is capable of replacing red light in every situation examined in which light promotes germination of lettuce seeds.

III. Brief irradiances of far red light, which reverse fully the potential promotion of germination by red light, do not negate gibberellin-mediated promotion of germination.

IV. Continuous far red light delays gibberellin-mediated promotion of germination, but under the conditions of these experiments, does not prevent germination permanently.

V. The promotive effects of gibberellin and red light are additive or less than additive when red light is administered 4 hours after seeds begin to imbibe on a gibberellin solution. When red light is administered 20 hours or longer after seeds are sown on gibberellin, the combined promotion of germination is greater than can be predicted by simple addition of the two single effects. These results suggest that a part of gibberellin action in light-sensitive seeds increases the responsiveness of the seeds to red light or maintains red light responsiveness at its maximum level. In lettuce seeds, this may be only a small part of the gibberellin effect, but in other seeds it may be the major or sole gibberellin action.

Acknowledgment

I thank Professor Anton Lang for his suggestions, advice, and criticism throughout this study.

Literature Cited

1. Borthwick, H. A., S. B. Hendricks, M. W. Parker, E. H. Toole, and V. K. Toole. 1952. A reversible photoreaction controlling seed germination. Proc. Natl. Acad. Sci. 38: 662–666.
2. Borthwick, H. A., S. B. Hendricks, E. H. Toole, and V. K. Toole. 1954. Action of light on lettuce-seed germination. Bot. Gaz. 115: 205–225.
3. Borthwick, H. A. and W. W. Robbins. 1928. Lettuce seed and its germination. Hilgardia 3: 275–305.
4. Bünsow, R. and K. von Bredow. 1958. Einfluss der Gibberelline auf die Tageslängenabhängigkeit der Samenkeimung von Kalanchoë. Naturwiss. 45: 95–96.
5. Evenari, M. 1952. The germination of letttuce seeds I. Light, temperature, and coumarin as germination factors. Palestine Jour. Bot., Jerusalem Ser. 5: 138–160.
6. Evenari, M. and G. Neumann. 1953. The germination of lettuce seed. III. The effect of light on germination. Bull. Res. Counc. Israel 3: 136–145.
7. Evenari, M., G. Neumann, S. Blumenthal-Goldschmidt, A. M. Mayer, and A. Poljakoff-Mayber. 1958. The influence of gibberellic acid and kinetin on germination and seedling growth of lettuce. Bull. Res. Counc. Israel 6D: 65–72.
8. Kahn, A. 1960. An analysis of "dark-osmotic inhibition" of germination of lettuce seeds. Plant Physiol. 35: 1–7.
9. Kahn, A., J. A. Goss, and D. E. Smith. 1956. Light and chemical effects on lettuce seed germination. Plant Physiol. 31 suppl.: xxxvii.
10. Kahn, A., J. A. Goss, and D. E. Smith. 1957. Effect of gibberellin on germination of lettuce seed. Science 125: 645–646.
11. Kribben, F. J. 1957. Die Abkürzung der Samenruhe bei Arabidopsis durch Gibberellinsäure. Naturwiss. 44: 313.
12. Lang, A. 1956. Gibberellin and flower formation. Naturwiss. 43: 544.
13. Lang, A. 1957. The effect of gibberellin upon flower formation. Proc. Natl. Acad. Sci. 43: 709–717.
14. Lona, F. 1956. L'acido gibberellico determina la germinazione dei semi di Lactuca Scariola in fase di scoto-inibizione. L'Ateneo Parmense 27: 641–644.
15. Poljakoff-Mayber, A., M. Evenari, and G. Neumann. 1958. Effect of red light and gibberellic acid on the temperature-inhibited germination of lettuce seeds. Bull. Res. Counc. Israel 6D: 99–102.
16. Thompson, R. C. and W. F. Kosar. 1939. Stimulation of germination of dormant lettuce seed by sulphur compounds. Plant Physiol. 14: 567–573.

TRANSPIRATIONAL SURGES IN TAMARIX AND EUCALYPTUS AS MEASURED WITH AN INFRARED GAS ANALYZER[1]

JOHN P. DECKER AND JANET D. WIEN[2]

ROCKY MOUNTAIN FOREST AND RANGE EXPERIMENT STATION, TEMPE, ARIZONA[3]

In many transpiration studies a method has been used in which a leaf or twig is severed from a plant and transferred quickly to a balance where subsequent weight losses are followed. Use of this method has been reviewed recently (1, 5). One objection to the method is that no information is obtained as to behavior of the leaf immediately before and after excision. Thus estimation of the quantity of most interest, transpiration rate of the intact leaf before excision, is difficult.

Andersson, Hertz, and Rufelt (1) developed an apparatus in which a corona-discharge hygrometer recorded the absolute humidity of air streaming over a single leaf. Thus they were able to obtain continuous records of transpiration before, during, and following excision of a leaf. With the species tested (*Solanum lycopersicum, Thunbergia alata, Citrus sp.*) transpiration increased rapidly immediately after excision and then declined. Their observations raise serious doubts about the usefulness of the cut-and-weigh method for estimating transpiration rates of intact leaves. In the present study, their tests were repeated with slightly different apparatus and two different species and with essentially similar results.

METHODS AND MATERIALS

APPARATUS: The apparatus (fig 1) consisted of a leaf chamber, a metered ventilation system, a sampling pump, and an infrared gas analyzer. A leaf or twig was sealed in the chamber and was ventilated at a known rate with dry air. Absolute humidity of air flowing from the chamber was recorded by the analyzer. Humidity, in milligrams per liter, multiplied by ventilation rate, in liters per minute, gave transpiration rate in milligrams per minutes.

The leaf chamber was the top of a 9.3 cm Petri dish sealed to a brass base plate with modeling clay. The rim of the dish was notched to accommodate a twig and a thermometer. Air temperature in the chamber was controlled automatically within 0.5° C with a thermo-regulator consisting of an ordinary mercurial thermometer to which was attached a Thermocap relay. The relay controlled a pump that forced water through a loop of tubing soldered to the lower side of the base plate.

Ventilation was continuous. Dry air flowed into the chamber from a high pressure cylinder (commercially compressed and dried air) through a reducing valve and floating-ball type flowmeter. Outflow from the chamber was through a large orifice around the thermometer stem.

The sampling pump (Thiberg model 1) withdrew air continuously at about 1,000 ml per minute from near the outlet orifice, passed it through the analyzer and returned it to the leaf chamber (fig 1).

The analyzer was a Liston-Becker model 15A infrared gas analyzer sensitized to water vapor and coupled to a recording milliammeter. Zero was set as the equilibrium reading for air recycled through a closed system consisting of the analyzer, pump, and a series of three drying tubes charged respectively with Drierite, Ascarite, and Anhydrone. Full scale was set as the equilibrium reading for air recycled through a bubbler of distilled water held at 20° ±0.01° C. The analyzer was checked against these two standards several times during each day of operation.

CALIBRATION: Calibration of the analyzer did not depend upon humidity tables. The procedure was as follows. A small, stoppered flask containing about 2 ml water was fitted with inlet and outlet tubes, and inserted into the system between the analyzer and empty chamber. The sample stream of air passed through the flask before returning to the chamber. Humidity at the outflow orifice became stable within a minute. Ventilation rate was adjusted to attain a selected humidity indication on the analyzer. The sampling pump was stopped, the flask was removed, and a weighed duplicate flask was substituted. The pump was run for 10.0 minutes, and the flask was removed and reweighed. Its loss of weight divided by the volume of ventilation gave average absolute humidity corresponding to that particular analyzer reading. With the chamber held at 34.5 to 35.5° C, duplicate observations were made over a series of five selected analyzer readings: corresponding average humidities were computed from weight losses; and a calibration curve was constructed (fig 2).

An earlier study with a closed-circuit apparatus (3) indicated a marked change of calibration with

[1] Received August 4, 1959.

[2] Present address: Botany Department, University of Arizona, Tucson.

[3] Forest Service, U. S. Department of Agriculture, general headquarters maintained at Fort Collins, Colo., in cooperation with Colorado State University. Senior author is stationed at Tempe, Ariz., in cooperation with Arizona State University.

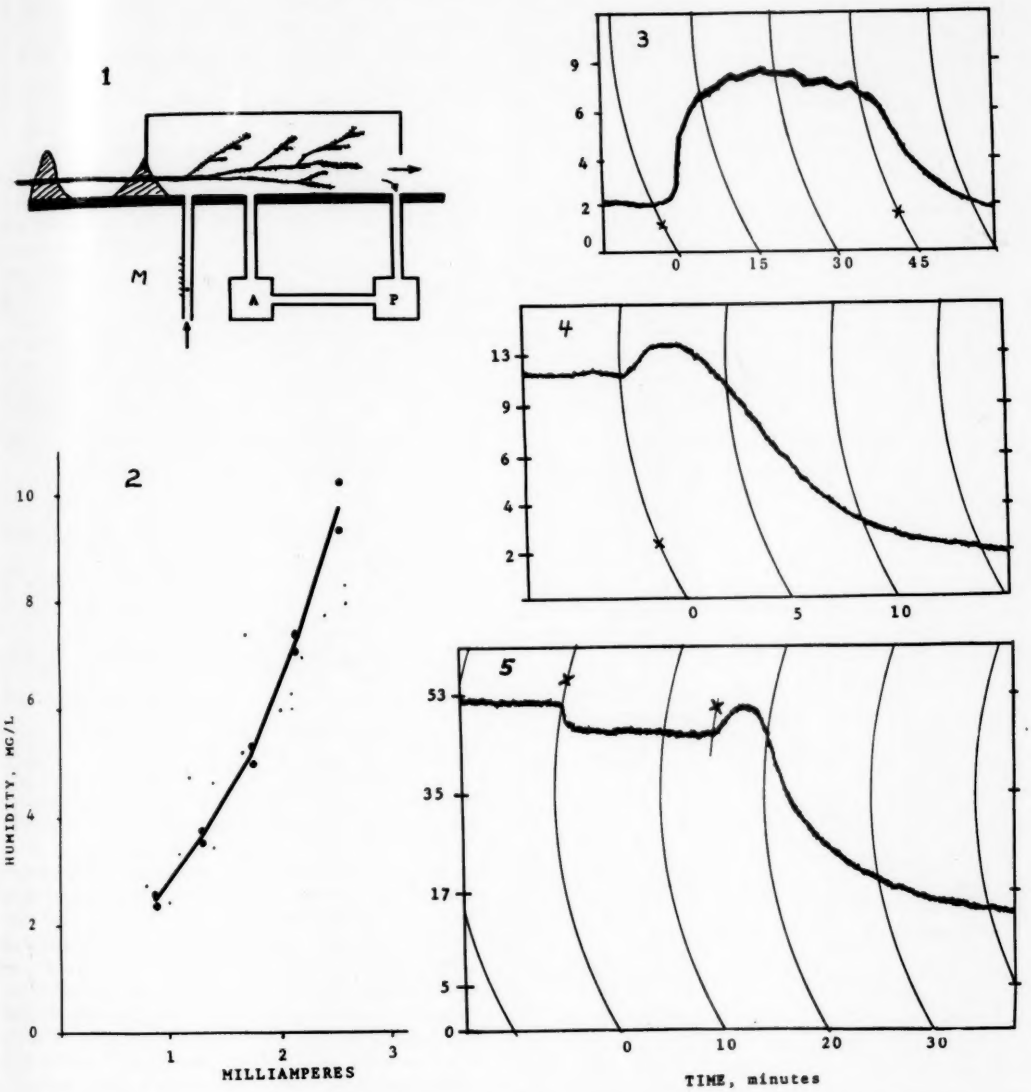


FIG. 1. Diagrammatic vertical section through transpiration chamber showing tamarisk twig installed under Petri dish cover. Clay cup at left was filled with water for certain experiments. Pump (P) and analyzer (A) are greatly simplified and reduced. Dry air for ventilation entered through flowmeter.

FIG. 2. Calibration curve for infrared gas analyzer based on observations at 35° C (large dots). Observations at 20° are indicated by small dots.

FIG. 3. Effect of light on time course of transpiration by an intact tamarisk twig at 35° C. Ordinate: transpiration rate in milligrams per minute. Abscissa: time in minutes. Lights on (3,000 ft-c, incandescent) at time 0, off at 45 minutes.

FIG. 4. Effect of cutting on time course of transpiration by a tamarisk twig at 35° C and 3,000 ft-c. Twig was cut in air with razor just outside chamber at time 0.

FIG. 5. Transpiration rate of eucalyptus leaf in milligrams per minute per dm^2 of leaf area. Petiole was cut under water with razor at time 0 and was exposed to air after 15 minutes.

temperature. To test the effect of temperature upon the present apparatus, the calibration procedure was repeated at another temperature (19.5–20.5° C). Results are shown as small dots in figure 2. The possible slight shift of the curve is of no importance to the present work at constant temperature, but it does suggest a need for caution in calibrating an apparatus to be used over a range of temperatures.

PLANTS: Ten potted seedlings of *Tamarix pentandra* Pall. and six of *Eucalyptus sp.* were used. The tamarisk plants were 80 to 100 cm tall and had grown in a greenhouse in 1-gallon cans of clay soil. They were transplanted to the cans as small seedlings (10–20 mm tall) dug from the banks of the Gila River near Phoenix, Ariz. The eucalyptus plants were 80 to 120 cm tall. They were ornamental planting stock purchased from a commercial nursery.

EXPERIMENTS AND RESULTS

STANDARD PROCEDURE: A twig of tamarisk or a leaf of eucalyptus was sealed in the chamber (fig 1) and was left under standard operating conditions (3000 ft-c of light, 34.5–35.5° C) until the transpiration rate became constant. Ventilation was then adjusted to bring the hygrometer reading conveniently near mid-scale, and experimental treatments were applied. Soil was kept near field capacity throughout the experiments.

DARK-LIGHT-DARK CYCLE: A well-known phenomenon, the change of transpiration rate with light and darkness, was used as a preliminary test of the apparatus. A tamarisk twig was arranged according to the standard procedure. The light was turned off until transpiration had dropped to a low and nearly constant rate. It was turned on until transpiration regained a constant high rate; then it was turned off again. Figure 3 is a photographic copy of a direct tracing of the milliammeter chart. The changes of transpiration were similar to those reported for other species (1). The procedure was repeated with eight other tamarisk twigs and eight eucalyptus leaves with similar results.

As has been previously suggested (1), the changes of transpiration probably resulted primarily from changes in stomata rather than from changes of leaf temperature. In an earlier study with a similar leaf chamber (2) a new stable leaf temperature was reached within the first 30 seconds of light or darkness, a change that is much too rapid to account for the changes of transpiration observed here.

TWIG OR PETIOLE CUT IN AIR: A tamarisk twig was arranged according to the standard procedure. It was then severed from the rest of the plant by cutting with a sharp razor just outside the chamber. Transpiration accelerated 13 % above the previous constant rate and then declined (fig 4). The procedure was repeated with 12 eucalyptus leaves and each showed a surge similar to that in figure 4. Surges ranged from 5 % to 30 % above the previous constant rate.

The surges were similar to those reported by Andersson, Hertz, and Rufelt (1) and in earlier works reviewed by them. A reasonable explanation for such surging that, according to them (1), was first suggested by Iwanoff is: water columns in the stem are under tension; cutting releases the columns, and for a short time water is drawn upward more freely.

The procedure was repeated with 29 other tamarisk twigs, with the cuts about 5 mm, 12 cm, or 25 cm from the chamber and made with either a sharp razor or dull wire-cutting pliers. Surges ranged from none to 23 %. No consistent relationship was apparent between height of surge and method or place of cutting. The behavior of tamarisk thus differed in this respect from that reported for tomato (1).

TWIG OR PETIOLE CUT UNDER WATER: A eucalyptus leaf was arranged according to the standard procedure. A small cup was formed of modeling clay around the petiole just outside the leaf chamber. The cup was filled with water and the petiole was severed under water with a sharp razor. Transpiration declined to a new constant rate (fig 5). The cup was then drained. Shortly after the cut end of the petiole was exposed to air, transpiration surged and declined (fig 5). The surge was about 9 % of the initial constant rate. The procedure was repeated with nine other eucalyptus leaves. All showed a similar pattern, with surges ranging from 9 % to 60 %.

The procedure was repeated wth eight more eucalyptus leaves except that the petioles were crushed off with round-jaw pliers instead of being cut with a sharp razor. With one leaf the response was similar to that shown in figure 5; with five leaves transpiration went into a continuous decline that was interrupted by a 9 to 26 % surge upon exposure of the cut end to air; with two leaves, transpiration went into a continuous and uneventful decline that was not affected by exposure to air.

The procedure was repeated with 24 tamarisk twigs, using a razor. With 13 twigs, transpiration declined smoothly and uneventfully; with six twigs, it began to decline, recovered slightly (before exposure to air), then went into a continuous decline; with five twigs, the pattern was similar to figure 5 with a 9 to 18 % surge.

The decrease of transpiration for eucalyptus and some tamarisk following cutting under water was similar to that reported for tomato (1) but was more definite and constant. A relationship to loss of root pressure has been suggested in explanation (1). Perhaps a simpler explanation is that crushing and tearing of xylary elements resulted in restricted lumina and increased resistance to flow of water.

The surge that followed subsequent exposure of a cut to air was similar to that reported by Glover (4). It may have occurred because air intruded more freely than water into damaged xylary elements. Alteration of response with method of cutting is further evidence that damage at the site of cutting was an important factor.

Summary

An intact twig of tamarisk or leaf of eucalyptus was sealed in an illuminated, constant-temperature glass chamber that was ventilated at a known rate with dry air. Absolute humidity of air flowing from the chamber was recorded continuously by an infrared gas analyzer. Humidity multiplied by ventilation rate gave transpiration rate. Transpiration increased smoothly to a new constant rate during illumination and decreased smoothly upon darkening. When a twig or petiole was severed in air, transpiration accelerated briefly in a smooth surge and then declined. Behavior was variable when the cut was made under water, but transpiration usually slowed to a new constant rate, then surged briefly when the cut was exposed to air.

Literature Cited

1. Andersson, N. E., D. H. Hertz, and H. Rufelt. 1954. A new fast recording hygrometer for plant transpiration measurements. Physiol. Plantarum 7: 753–767.
2. Decker, J. P. 1955. A rapid, postillumination deceleration of respiration in green leaves. Plant Physiol. 30: 82–84.
3. Decker, J. P. and B. F. Wetzel. 1957. A method for measuring transpiration of intact plants under controlled light, humidity, and temperature. Forest Sci. 3: 350–354.
4. Glover, J. 1941. A method for the continuous measurement of transpiration of single leaves under natural conditions. Ann. Bot. (N. S.) 5: 25–34.
5. Parker, J. 1957. The cut-leaf method and estimations of diurnal trends in transpiration from different heights and sides of an oak and a pine. Bot. Gaz. 119: 93–101.

SEPARATION AND IDENTIFICATION OF SOLUBLE NUCLEOTIDES FROM ETIOLATED CORN SEEDLINGS AS A FUNCTION OF GROWTH[1, 2]

JOE H. CHERRY[3] AND R. H. HAGEMAN

Department of Agronomy, University of Illinois, Urbana

Nucleotides comprise one of the most active, varied, and versatile groups of compounds in nature. Nucleotide polymers, combined with specific proteins appear to be one of the prerequisites of living matter. Nucleotides participate in and regulate all phases of metabolism: carbohydrate, lipid, protein, and nucleic acid. Many processes take place only when the substrate has been activated by combination with a nucleotide. In addition, nucleotides have the major role in trapping, conserving, and transferring energy in living systems.

Henderson and LePage (7) have recently reviewed numerous studies involving isolation and characterization of naturally occurring acid soluble nucleotides. Most of the known nucleotides were isolated from microbial and animal tissues. Harris et al (6) employing a double extraction method, have reported that yeast cells showed an increase and diversification of nucleotides when extracted during the period prior to cell division. In contrast, fewer investigations have been made of the nucleotides from higher plants.

Albaum et al (1, 2) succeeded in isolating and identifying ADP[4] and ATP from mung bean seedlings. Later, Ginsburg et al (5) isolated the adenosine nucleotides as well as the uridine diphosphate sugar derivatives from mung beans. Solms and Hassid (11) identified uridine diphosphate *N*-acetylglucosamine and uridine diphosphate glucuronic acid as constituents of the same material. Rowan (10) extracted ADP, ATP, UDP, and UTP from pea seedlings and identified them by use of paper chromatography and specific enzyme assays.

---

[1] Received August 10, 1959.

[2] Contribution from the Department of Agronomy. Part of a thesis submitted by the senior author in partial fulfillment of the Masters requirement. Supported by Grant No. 4407 from the National Science Foundation and by federal funds granted by the Hatch Act.

[3] Current address: Department of Agronomy, University of Illinois, Urbana.

[4] The following abbreviations will be used: AMP, adenosine monophosphate; ADP, adenosine diphosphate; ATP, adenosine triphosphate; UMP, uridine monophosphate; UDP, uridine diphosphate; UTP; uridine triphosphate; GMP, guanosine monophosphate; GDP, guanosine diphosphate; GTP, guanosine triphosphate; CMP, cytosine monophosphate; CDP, cytosine diphosphate; CTP, cytosine triphosphate; TPN, triphosphopyridine nucleotide; DPN, diphosphopyridine nucleotide; PCA, perchloric acid.

The work reported in this paper was undertaken to provide a systematic separation and identification of soluble nucleotides from higher plants. The principle objectives were to identify the predominant nucleotides and to determine relative changes in concentration of the nucleotides as a function of growth in etiolated corn (*Zea mays* L.) seedlings.

Materials and Methods

Plant Material: Plant material for nucleotide assays was obtained from seedlings of the single-cross corn hybrid (WF9 × M14) seeds[5]. After treating with Spergon (tetra chloro-para-benzoquinone) 120 seeds were placed embryo down on paper toweling. The paper toweling was supported by a layer of acid washed gravel (600 ml) in a 3 qt Pyrex utility dish. After adding 260 ml 10^{-4} M $CaCl_2$, each dish was covered with Saran-wrap. Small holes were punched in the Saran cover to provide aeration. The seeds were germinated in a dark, aerated germinator at a temperature maintained at 29° C with the relative humidity at 75 % or higher. Additional $CaCl_2$ solution was added to the dish as needed when seeds were germinated beyond 48 hours. Vigorous and uniform seedlings were obtained routinely with these techniques. In general, 7- and 8-day old seedlings exhibited symptoms of deterioration and therefore were not used for experimental material. Experience in this laboratory has shown that plants grown under the described conditions deplete the endosperm tissue by the seventh or eighth day. Seedlings were harvested at one through six days of age. Nucleotides were extracted from the tissue, chromatographed, and identified. The entire procedure was repeated three times for each of the six growth stages.

Extraction of Soluble Nucleotides: At the time of sampling, the endosperm was removed from each seed; only the embryonic tissue (scutellum and embryonic axis) was used for assay. The extraction procedure described by Hurlbert et al (8) was used with the following modifications. Tissue was kept ice-cold prior to blending in an Omni-mixer for 5 minutes at maximum speed; only one extraction was made with cold 0.6 *N* PCA; 40 gm plant tissue (fresh weight) was used for each extraction.

Ion-Exchange Separation Procedure: The procedure used for chromatographic separation of nucleotides was similar to that described by Hurlbert et al (8), but was modified to provide a more nearly linear elution gradient. Figure 1 is a diagram of the modified elution apparatus. The two-reservoir elution principle was patterned from the system designed by Dr. A. H. Beavers (Agronomy Department, University of Illinois, Urbana) for preparing a density gradient column. The two reservoirs used were modified 1 liter Pyrex Erlenmeyer flasks. Reservoir A contained a mixture of 4.0 *N* formic acid–1.6 M ammonium formate. Reservoir B contained only 4.0 *N* formic acid. Initially the mixing flask contained 500 ml deionized water. The column (1.2 × 60 cm) was packed to a height of 40 cm with Dowex-1 × 10 (200-400 mesh, formate form). The elution was started by allowing 500 ml of 4.0 *N* formic acid (reservoir B) to pass through the system. Reservoirs A and B were then filled to the same level with their respective eluents and both stopcocks were opened.

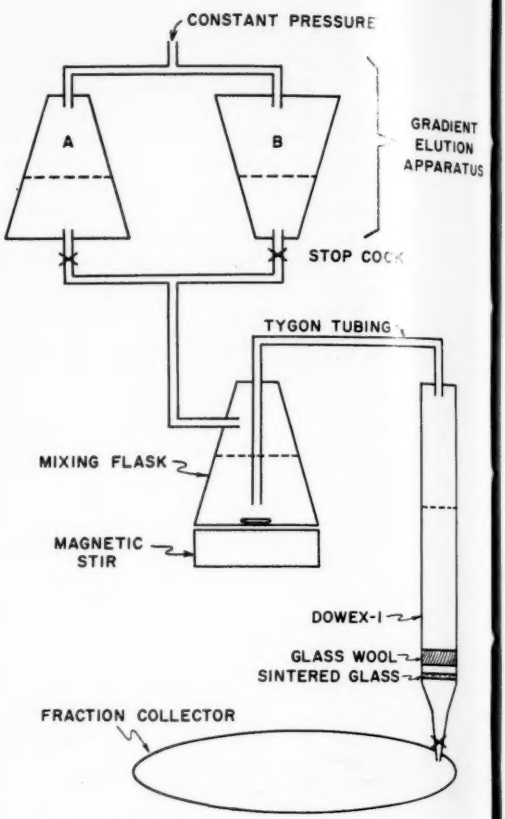


Fig. 1. Ion-exchange chromatography separation equipment. A Rinco (GM Instrument Co., Greenville, Ill.) volumetric fraction collector was used to collect the samples.

Five ml fractions were collected. Nucleotide content in each tube was determined by measuring the light absorption at 260 mμ in a Beckman Spectrophotometer. An elution chromatogram was made by plotting light absorption (260 mμ) against tube number.

Purification of Nucleotide Fractions: Eluates from each peak were combined and mixed with

[5] The seed graded "medium flat" was supplied through the courtesy of Dr. W. J. Mumm, Crows Hybrid Corn Company, Milford, Ill.

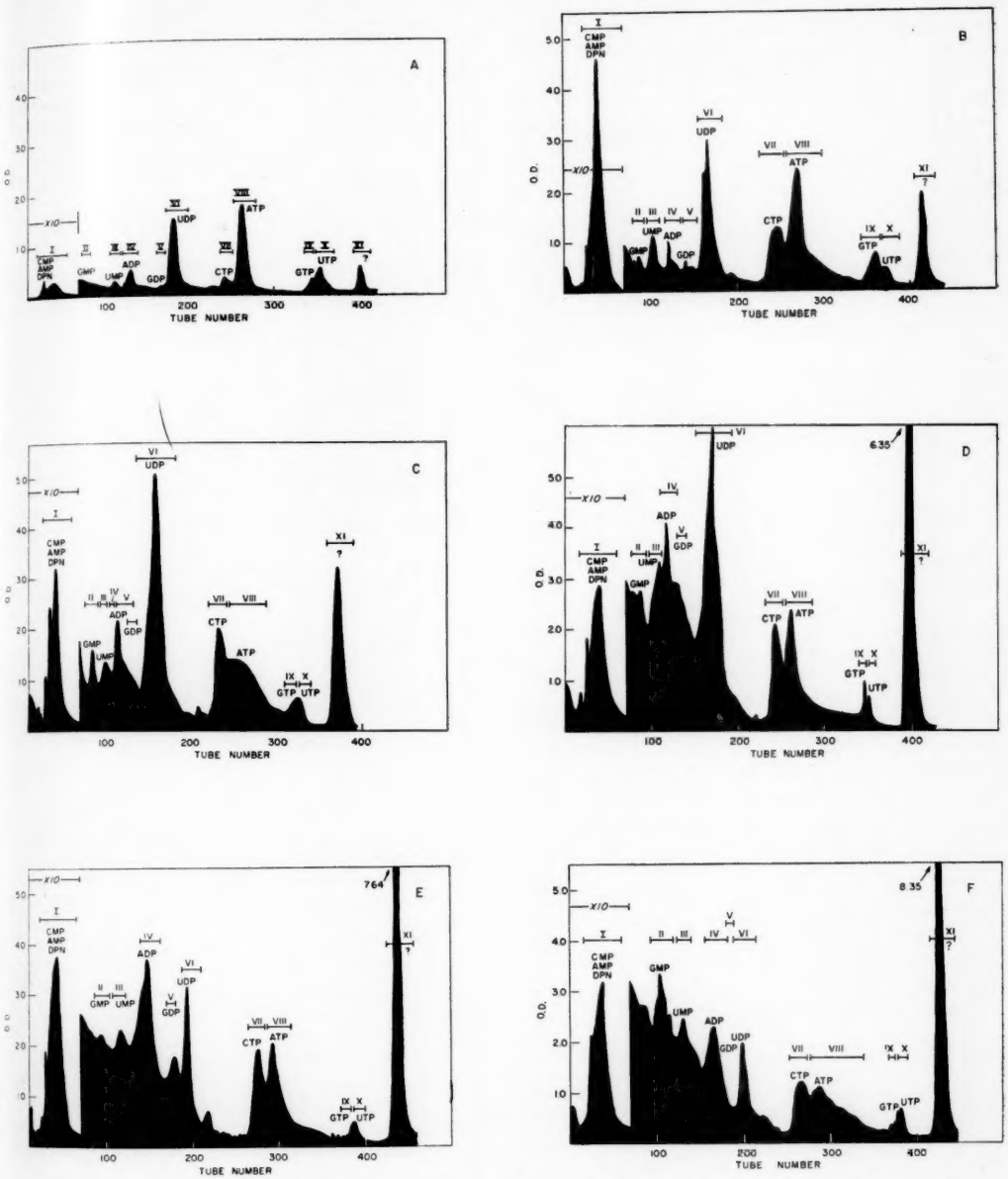


FIG. 2. Ion-exchange elution-chromatograms from corn tissue 1 through 6 days of age (A through F, resp.). The values (O.D.) represent the nucleotide content from 10 gm of dry seedling material. The X10- indicates that the values plotted for the first 60 tubes are 1/10th the actual values.

0.3 gm activated charcoal (Darco grade G60) and stirred for 5 minutes to allow the charcoal to adsorb the nucleotides. The charcoal (with the adsorbed nucleotides) was removed by filtering through Whatman No. 1 paper and washed with four 10 ml volumes of cold water to remove salts and other impurities. Nucleotides were then eluted from the charcoal with two 25 ml portions of aqueous pyridine (15%). The pyridine solution containing the nucleotides was placed in a 150 ml separatory funnel; the pyridine was extracted with four 15 ml volumes of chloroform. This chloroform-pyridine solution was discarded. The aqueous nucleotide solution was then lyophilized to dryness, dissolved in 2 ml of water and used for subsequent identification.

IDENTIFICATION: An ultra-violet adsorption spectrum covering the region between 220 and 300 mμ was determined for each purified fraction at pH 2 and 7. The ultra-violet spectra and the ratios of absorbancy at 250 to 260 mμ and 280:260 mμ were compared with spectra and ratios of pure nucleotides (3, 9).

Each purified peak was co-chromatographed on Whatman No. 3 paper with standard 5′ nucleotides (sodium salt form, purchased from Pabst). Paper chromatographic data were obtained using Pabst solvents I, II, and III (9).

Identity of the di- and triphosphate nucleotides was established by paper electrophoresis. Acid-washed (acetic) Whatman No. 3 paper was stitched with 24 gauge copper wire one-half inch from each end of the paper. Each stitch was placed in and out of the paper every half inch. Unknown and standard nucleotides were spotted on the paper and suspended in a 3 qt Pyrex utility dish by the copper wire. Uniform contact between the paper and the wire was made by a NaCl-Agar mixture. The paper was sprayed with 20 ml 4.6% *N* butyric acid (pH 3.2). A glass plate was placed over the dish and the nucleotides were allowed to migrate toward the anode for 2 hours with the current maintained at 4 ma with 250 volts using a Reco power supply (Model E 800-2; Research Equipment Corp., Oakland, Calif.) After the paper was removed and dried, the nucleotides were located with an ultra-violet Mineralight (Model SL 2537).

RESULTS AND DISCUSSION

I. ISOLATION AND IDENTIFICATION: One-day old corn seedlings were chosen as the source of material for separation and identification of the acid soluble nucleotides. This material was selected because the older tissue contained a greater percentage of material absorbing at 260 mμ that eluted from the column in the first portion of the chromatogram (0-60 tubes). For this reason the nucleotides from the same quantity (fresh weight) of 1-day tissue, separated more sharply throughout the entire chromatogram. Even with 1-day old seedlings complete separation of the monophosphate nucleotides, DPN, and TPN was not obtained. Eleven separate nucleotide fractions were selected as indicated by the Roman numerals of the Dowex-1 elution-chromatogram (fig 2A) from 1-day old corn seedlings. The Dowex-column chromatography did not give complete separation of the nucleotides. A comparison of figure 2 and table II indicates a cross-continuation in some peaks, for this reason subsequent purification and paper chromatographic and electrophoretic separations were used for final identification. The data were recomputed to represent the quantity of nucleotides extracted from 10 gm dry material as well as the content per 100 seedlings.

Data identifying each of the 11 nucleotide fractions are presented individually.

PEAK I. At least four nucleotides were present in the composite of fraction I (Peak I). No attempt was made to determine the exact amount of each component. Since this fraction was eluted from the

TABLE I

PAPER ELECTROPHORETIC AND PAPER CHROMATOGRAPHIC PROPERTIES OF NUCLEOTIDES FROM CORN SEEDLINGS AND OF AUTHENTIC NUCLEOTIDES USED FOR COMPARISON

SAMPLE	PAPER ELECTROPHORESIS[1] CM MIGRATED	PAPER CHROMATOGRAPHY (SOLVENT III)[2] R_f VALUE
Ia	...	0.24
AMP	...	0.28
DPN	...	0.32
Ib	...	0.42
TPN	...	0.45
Ic	...	0.59
Id	...	0.68
CMP	...	0.74
IIa	...	0.32
IIb	...	0.57
GMP	...	0.55
III	6.4	0.71
UMP	6.3	0.72
IV	5.8	0.33
ADP	5.4	0.33
V	...	0.67
GDP	...	0.60
VI	11.0	0.75
UDP	11.0	0.75
VII	9.0	0.80
CTP	12.0	0.80
VIII	8.5	0.33
ATP	8.8	0.35
IX	11.0	0.52
GTP	11.5	0.52
X	15.0	0.71
UTP	16.0	0.70
XI	19.0	0.25

[1] All values are for a 2 hour period (4 ma and 250 volts) using a 4.6% *n*-butyric acid spray as the solvent.
[2] Solvents I and II verified results obtained from solvent III.

column prior to adding ammonium formate, the samples were lyophilized directly to dryness. This eliminated the charcoal purification step (methods) and the partial loss of nucleotides inherent in this procedure.

The lyophilized material was dissolved in a small volume of water and aliquots were chromatographed on Whatman No. 3 paper. Solvent III proved more satisfactory than I and II for separation of the nucleotide components and was used most extensively.

When the chromatograms were developed, four areas (Ia, Ib, Ic, and Id) were observed which absorbed ultra-violet light. The area nearest (Rf value of 0.22–0.28) the origin was a large diffuse spot (Ia). It was similar in migration characteristics to standard AMP and DPN (table I). An area of the chromatogram which contained this spot was cut out and the components eluted from it with water. The spectral characterization (table II) of the eluate indicated that the components could be either AMP or DPN. An aliquot of the eluate was treated with sodium hydrosulfite and the change in absorbancy at 340 mμ was observed. An increase in absorption after reduction, confirmed the presence of DPN. From these data it was concluded that the spot (Ia) contained a mixture of AMP and DPN. The amount of DPN present in the combined eluant of Peak I was relatively small in comparison to the two nucleotides, AMP and CMP.

The second area (Ib) had an Rf value of 0.42 (table I) which is similar to that of standard TPN. The spectral data obtained in subsequent tests, however, did not confirm this identity.

The third area (Ic) had an Rf value of 0.59 (table I) which is similar to standard GMP, but this area was observed only in a few instances. In these cases, the presence may result from overlapping of peaks I and II.

The fourth spot (Id) had an Rf value of 0.68 which is similar to the Rf value obtained from standard

TABLE II

ULTRAVIOLET ABSORPTION OF NUCLEOTIDES FROM CORN SEEDLINGS AND OF AUTHENTIC NUCLEOTIDES USED FOR COMPARISON

SAMPLE	MAX mμ pH		MIN mμ pH		ABSORBANCY RATIOS AT 250, 260, AND 280 mμ			
					250/260 pH		280/260 pH	
	2	7	2	7	2	7	2	7
Ia	....	258	....	236	....	0.90	....	0.43
AMP	....	260	....	230	....	0.80	....	0.15
DPN	....	260	....	235	....	0.76	....	0.10
TPN	....	260	....	235	....	0.74	....	0.10
Id	....	275	....	250	....	0.87	....	0.91
CMP	....	275	....	249	....	0.86	....	0.98*
II	260	255	235	235	0.88	0.98	0.81	0.69
GMP	256*	252	228*	230	0.96*	1.13	0.67*	0.63
III	260	258	235	235	0.87	0.94	0.58	0.55
UMP	262*	262	230*	230*	0.73*	0.67	0.39*	0.34
IV	258	260	232	230	0.88	0.86	0.33	0.34
ADP	257*	260	230	230	0.85*	0.78	0.21*	0.16
V	256	256	235	235	0.97	0.59	0.97	0.59
GDP	256*	255	228*	224*	0.95*	1.15*	0.67*	0.59
VI	260	260	230	230	0.80	0.81	0.41	0.41
UDP	262	262	230	230	0.80	0.73	0.39	0.39
VII	270	275	242	250	0.89	0.86	1.00	1.37
CTP	280*	271	241	249	0.45	0.84	2.12	0.97
VIII	260	260	230	230	0.84	0.82	0.26	0.20
ATP	257*	260	230	230	0.85*	0.78	0.22*	0.14
IX	255	255	225	225	1.03	1.15	0.61	0.69
GTP	256*	252	228*	223	0.96*	1.14	0.67*	0.66
X	....	261	....	230	....	0.77	....	0.43
UTP	....	262	....	230	....	0.76	....	0.40
XI	270	270	245	242	0.80	0.80	0.92	0.85

* Spectrophotometric constants of 5′ ribonucleotides were obtained from Pabst (9).

CMP. Co-chromatography and spectral characteristics identified this component as CMP.

PEAK II. The composite of this fraction was purified with charcoal, lyophilized to dryness, redissolved in water; aliquots were used for identification. Each subsequent fraction (III through XI) was treated in the same way to remove the ammonium formate and concentrate the sample. Paper chromatography revealed that in most cases there were two nucleotide components present in Peak II. Data presented in tables I and II identify the major component (IIb) as GMP. Additional confirmation was obtained by separation of a mixture of four standard monophosphate nucleotides (CMP, AMP, GMP, and UMP) on the Dowex-column. The standard GMP was eluted from the column at the same tube range (between tubes 80–90) as Peak II.

Where a second component (IIa) was observed the Rf values (table I) and spectral characteristics (table II) indicated the presence of AMP.

The occasional presence of GMP in Peak I and the detection of AMP in Peak II demonstrate the failure of the Dowex-column to sharply separate these fractions. Figure 2 shows that Peak II-GMP is actually a trailing shoulder of Peak I.

PEAKS III, IV, VI, VII, VIII, IX, AND X. The Dowex-column gave good separation of the remaining nucleotides (fig 2A). Positive identifications of each of the purified fractions were obtained by co-chromatography (table I) and ultra-violet absorption (table II) characteristics. They were identified as follows: III-UMP, IV-ADP, VI-UDP, VII-CTP, VIII-ATP, IX-GTP, and X-UTP.

PEAK V. Positive identification of this peak was not obtained. The location (tube number) on the elution chromatogram (fig 2A) suggests that the component is GDP; however, this was not confirmed by chromatographic data (table I) or spectral data (table II).

PEAK XI. This fraction which has not been identified was the last component to be eluted from the Dowex-column (fig 2A). Its position in the elution-chromatogram indicates that the component possesses a stronger net negative charge than the triphosphate nucleotides. The chromatographic and electrophoretic data (tables I and II) confirmed the negative character of this component, but failed to reveal any similarity to known nucleotides. All data indicated that the fraction contained a single component. This compound is not a nucleotide, since phosphorus analysis (4) reveals that P is not an elemental constituent.

Preliminary trials indicate that this compound (2.0 mg/15 ml) inhibits tissue respiration and growth of root-tip sections. Additional work is underway to establish the identity and metabolic function of this compound.

II. CHANGES IN NUCLEOTIDE CONTENT AS FUNCTION OF GROWTH: Changes in nucleotide content as a function of growth were determined from young (1 through 6 days) corn seedlings. The procedure for harvesting, extraction, column separation, and identification has been described previously. Elution-chromatogram pattern (fig 2A) which was obtained from 1-day old seedlings, was used extensively as a guide in identifying the nucleotide components in subsequent elution chromatograms of the older seedling material. Final identity of the nucleotides was confirmed in all cases by electrophoresis, chromatography, or spectral characterization.

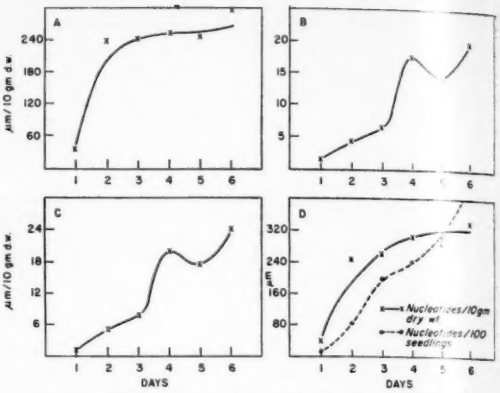


FIG. 3. Changes in the content of monophosphate nucleotides and derivatives from corn seedlings as a function of growth. Changes in nucleotide content of fractions I (CMP-AMP-DPN), II (AMP-GMP), and III (UMP) are illustrated in 3A, 3B, and 3C, respectively, and represent the nucleotides from 10 gm (dry weight) seedling material. The sum of the nucleotide content (10 gm dry weight and 100 seedling) of the three fractions (I, II, and III) is plotted in 3D.

Elution chromatograms of the acid soluble nucleotides extracted from young (1 through 6 days) corn seedlings are shown in figure 2 (A, B, C, D, E, and F). Each of the six elution chromatograms represents the average values from three separate lots of seedling material. Variations were inherent in the degree of separation, location (tube number) of the fraction, and amounts (absorption at 260 mμ) of nucleotides among the replicates. Mechanical failure of the fraction collector was responsible for some of the variability.

The amount of nucleotide in each fraction is directly proportional to the area under the curve (fig 2). This amount was computed by summing the product (volume of eluate × absorption at 260 mμ × molar absorbancy of each nucleotide) of the tubes composited from each fraction. Molar absorbancy values of AMP and GMP were used in computing Peaks I and II, respectively, where more than one nucleotide were present. The absorbancy coefficient of CTP was chosen for the computation of compound XI content because of the similarity in absorption

spectra. The molar absorbancy constants at pH 2 given by Pabst (9) were used. These amounts were expressed as micro-moles of nucleotide per 10 gm of dry seedling material or per 100 seedlings.

Changes that were observed as a function of growth will be discussed individually for each fraction.

PEAK I—CMP, DPN, AND AMP. Peak I contained the nucleotides CMP, DPN, and AMP. Although this peak was not separated into its component parts, it is believed on the basis of preliminary tests that the first serration on the shoulder of this peak was CMP; the remaining portion was a mixture of DPN and AMP.

The changes in nucleotides or nucleotide derivatives per 10 gm (dry weight) of seedling material as a function of growth are illustrated in figure 3A. The nucleotide content of fraction I (CMP, DPN, AMP, and related compounds) increased 650 % during the second day of germination. Little change in nucleotide content was observed in the seedling material between the second and fifth days while the oldest (6 day) material contained the maximum nucleotide content.

PEAK II—GMP. Figure 3B illustrates the change in GMP content with increased seedling age. This fraction gradually increased in content until the seedlings were 3 days old then increased sharply, from the 3rd to the 4th day. The GMP content dropped on the 5th day and rose to a maximum level on the 6th day.

PEAK III—UMP. The UMP content per 10 gm (dry weight) seedling material had a development pattern similar to that of GMP. This is illustrated in figure 3C.

TOTAL MONOPHOSPHATE NUCLEOTIDES AND NUCLEOTIDE DERIVATIVES: The sums of the nucleotides of the first three fractions (Peaks I through III) are plotted as a function of seedling growth (fig 3D). These data were plotted as total micro-moles of nucleotides or derivatives per 10 gm (dry weight) of seedling material and content per 100 seedlings. The monophosphate nucleotides computed on a per plant basis increased in a linear manner with seedling age whereas on a dry weight basis the increase was curvilinear.

PEAK IV—ADP. This fraction was identified as ADP and no other component was detected when the purified material was chromatographed. This peak was the first of the diphosphate nucleotides to be eluted from the Dowex-column (fig 2) in detectable quantities. The quantity of ADP in the seedlings increased gradually over the first 5 days (fig 4A) and then dropped sharply.

PEAK V—GDP. The component in the composited eluant of Peak V was not conclusively identified as GDP. As shown in figure 4B only small quantities of compound were obtained from the Dowex-column. The GDP concentration increased during the 2nd and 3rd days reaching a maximum on the 4th day and decreasing rapidly in the 5th and 6th day-old seedling material.

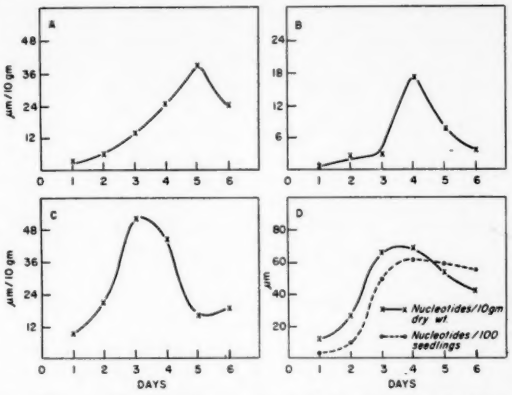


FIG. 4. Changes in content of diphosphate nucleotides from corn seedlings as a function of growth. Changes in nucleotide content of fractions IV (GDP), V (ADP), and VI (UDP) are illustrated in 4A, 4B, and 4C, respectively, and represent the nucleotides from 10 gm (dry weight) seedling material. The sum of the nucleotide content (10 gm dry weight and 100 seedlings) of the three fractions (IV, V, and VI) is plotted in 4D.

PEAK VI—UDP. Concentration of UDP in the seedlings increased rapidly during the 2nd and 3rd days of growth but later decreased sharply in older seedling material (fig 4C).

TOTAL DIPHOSPHATE NUCLEOTIDES: The sums of the diphosphate nucleotide fractions (V, VI, and VII) as a function of growth are illustrated in figure 4D. The total diphosphate nucleotide content per 10 gm (dry weight) of seedling material increased during the first 3 days of germination whereas the content decreased in the older seedling material. The diphosphate nucleotide content on a per seedling basis (100 plants) increased until the 4th day and decreased slightly thereafter.

PEAK VII—CTP. Peak VII was identified as CTP. There was an overlapping of Peaks VII and VIII (fig 2), however, the CTP content increased as a function of growth through the first 4 days and decreased on the 5th and 6th days (fig 5A)

PEAKS VIII—ATP. The nucleotide component of this fraction was confirmed as ATP. In the earlier (1 through 2 days) stages of seedling growth, ATP was the predominant triphosphate nucleotide present (fig 2). ATP content of the seedlings increased 400 % from 1 to 2 days and decreased slightly in the subsequent stages of seedling development (fig 5B).

PEAK IX—GTP. This fraction was eluted from the column just prior to Peak X, with some overlapping of these fractions. It was identified as GTP.

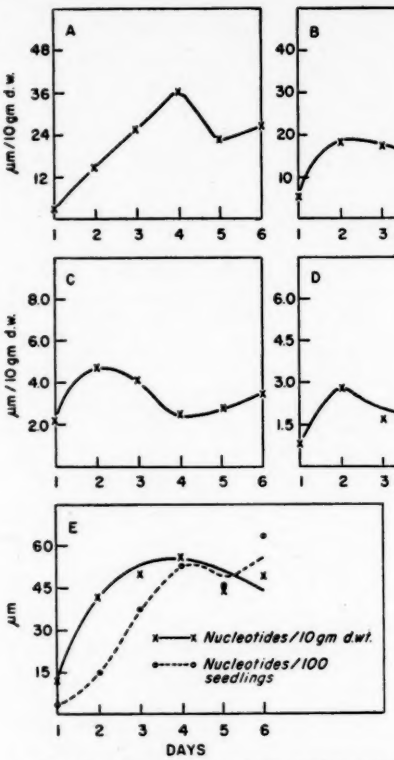


FIG. 5. Changes in content of triphosphate nucleotides from corn seedlings as a function of growth. Changes in nucleotide content of fractions VII (CTP), VIII (ATP), IX (GTP), and X (UTP) are illustrated in 5A, 5B, 5C, and 5D, respectively, and represent the nucleotides from 10 gm (dry weight) seedling material. The sum of the nucleotide content (10 gm dry weight and 100 seedlings) of the four fractions (VII, VIII, IX, and X) is plotted in 5E.

The GTP content (Peak IX) reached a maximum at the 2nd day; it decreased as a result of subsequent growth (fig 5C).

PEAK X—UTP. UTP content per 10 gm (dry weight) of seedling material had a development pattern similar to Peak IX-GTP. This is illustrated in figure 5D.

TOTAL TRIPHOSPHATE NUCLEOTIDES: Total triphosphate nucleotide (Peaks VII, VIII, IX, and X) content changed (fig 5E). The total triphosphate nucleotide content in the seedling material (10 gm dry weight or per 100 seedlings) increased with germination through the first 4 days. Very little change in triphosphate nucleotide content was observed from the 4th to the 6th days of development.

PEAK XI. The amount of compound (XI) per 10 gm of material, arbitrarily computed as a nucleotide, increased progressively with growth during the first 4 days. No further changes in content were observed. In contrast, the amount of this compound per 100 seedlings (fig 6) increased throughout the 6 days of growth.

TOTAL NUCLEOTIDES: Changes in the total nucleotides (Peaks I through XI) are illustrated in figure 7. Total nucleotide content in the seedling material (10 gm dry weight) increased throughout the 6 days of growth. The greatest increase in total nucleotide content occurred between the 1st and 2nd days of growth. Total nucleotide content per 100 plants increased in a linear manner throughout the 6 days of growth.

III. RATIOS OF NUCLEOTIDES (MONO-: DI-: TRIPHOSPHATE) AS FUNCTION OF GROWTH: The changes in ratio of the mono-: di-: triphosphate of uridine, di-: triphosphates of adenosine and the total mono-: di-: triphosphate nucleotides are presented in table III.

RATIO OF URIDINE NUCLEOTIDES: UDP was the predominate uridine nucleotide in the early stages of growth (1 through 3 days). UMP increased steadily until the 6th day when it was the most abundant uridine nucleotide. The ratio of UTP to UDP plus UMP changed from 1:10 to 1:39 during the first 3 days of growth and decreased thereafter.

RATIO OF ADENOSINE NUCLEOTIDES: Since the quantitative content of AMP from corn seedlings was not determined, only ratios of ADP:ATP were computed. The ADP:ATP ratio increased gradually until it reached the maximum value on the 5th day and they declined to 1.4:1 on the 6th day.

RATIO OF TOTAL NUCLEOTIDES: The ratios of total mono-:di-:triphosphate nucleotides that were

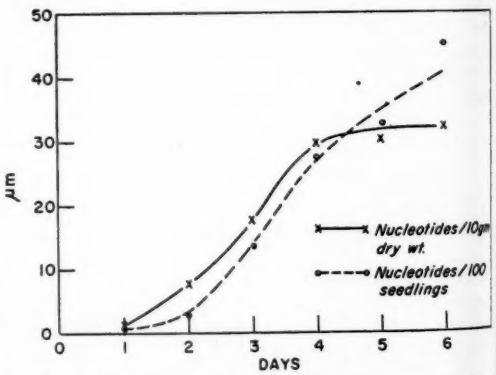


FIG. 6. Changes in the content of compound XI as a function of growth. Data were obtained from ultraviolet light absorption (260 mµ) and computed on basis of 10 gm of dry seedling material and content per 100 seedlings.

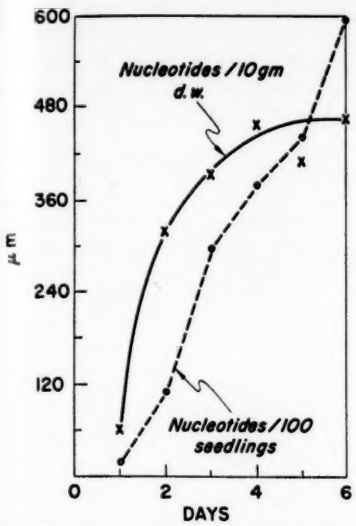


FIG. 7. Change in total nucleotide content per seedling material as a function of growth. Data were obtained from ultra-violet absorption (260 mμ) and computed on basis of 10 gm of dry seedling material, and content per 100 seedlings.

computed show an increasing value for the total monophosphate nucleotides with increasing seedling age. This suggests a conversion from the higher energy di- and triphosphate nucleotides concomitant with growth. This conversion appears to be primarily at the expense of the diphosphate nucleotides (figs 3D, 4D, and 5E), after the seedlings were 4 days old.

SUMMARY

The acid soluble nucleotides from etiolated corn seedlings have been isolated and identified using ion-exchange chromatography, paper chromatography, paper electrophoresis, spectral analysis, and chemical analysis. Positive identification of AMP, ADP, ATP, DPN, CMP, CTP, UMP, UDP, UTP, GMP, and GTP from corn seedlings has been made. The presence of GDP and TPN was indicated. In addition to the identification of the commonly occurring nucleotides a new compound (Peak XI) has been isolated but not identified. Although this compound absorbs ultra-violet light it is not a nucleotide.

Change in nucleotide content (per 10 gm dry weight) as a function of growth was determined. Monophosphate nucleotides (AMP, UMP, and CMP) and DPN per unit of dry material increased rapidly during the first 4 days of germination, but remained nearly constant during the last 2 days.

Diphosphate nucleotide (ADP, UDP, and GDP) content increased with seedling growth through the 3rd day of germination, remained constant on the 4th day, and decreased thereafter.

Triphosphate nucleotides (ATP, CTP, GTP, and UTP) increased through the first 4 days of germination and then remained at about the same level the last 2 days.

The content of the new compound (Peak XI) per unit of dry material increased 15 fold from 1 to 4 days of germination with no apparent increase following subsequent seedling growth.

Total nucleotide content increased in a curvilinear manner when plotted per unit of dry material (10 gm), but was linear when plotted on basis of content per 100 seedlings.

The ratios of mono-: di-: triphosphate nucleotides as a function of growth were computed. These data indicate a gradual shift from the high energy di- and triphosphate nucleotides to the monophosphate nucleotides with germination of the etiolated seedlings.

LITERATURE CITED

1. ALBAUM, H. G. and M. OGUR. 1947. An adenine-pentose-pyrophosphate from plant tissue. Arch. Biochem. Biophys. 15: 158–160.
2. ALBAUM, H. G., M. OGUR, and A. HIRSHFELD. 1950. The isolation of adenosine triphosphate from plant tissue. Arch. Biochem. Biophys. 27: 130–142.
3. CHARGAFF, ERWIN and J. N. DAVIDSON. 1955. The Nucleic Acids. Vol. I: 692 pp. and Vol. II: 576 pp. Academic Press, Inc., N. Y.
4. FISKE, C. H. and Y. SUBBAROW. 1925. The colorimetric determination of phosphorus. Jour. Biol. Chem. 66: 375–400.

TABLE III

RATIO OF MONO-: DI-: TRIPHOSPHATE NUCLEOTIDES FROM CORN SEEDLINGS AS FUNCTION OF GROWTH*

NUCLEOTIDE RATIO	AGE OF SEEDLING MATERIAL DAYS					
	1	2	3	4	5	6
UMP: UDP: UTP	1:9:1	2:7:1	5:34:1	11:25:1	8:8:1	13:10:1
ADP: ATP	0.4:1	0.3:1	0.8:1	1.5:1	2.5:1	1.4:1
Mono-: di-: Triphosphate Nucleotides**	3:1:1	6:0.6:1	5:1:1	5:1:1	7:1:1	7:1:1

*Values were computed on basis of 10 gm dry seedling material.
**The total mono- (including Peak I and therefore AMP), di-, and triphosphate nucleotides were used for these computations.

5. GINSBURG, V., P. K. STUMPF, and W. Z. HASSID. 1956. The isolation of uridine diphosphate derivatives of D-glucose, D-galactose, D-xylose and L-arabinose from mung bean seedlings. Jour. Biol. Chem. 233: No. 2, 357–364.
6. HARRIS, G., J. W. DAVIS, and R. PARSONS. 1958. Nucleotide pool of brewer's yeast during growth and fermentation. Nature. 182: 1565–1567.
7. HENDERSON, F. J. and G. A. LEPAGE. 1958. Naturally occurring acid-soluble nucleotides. Chem. Rev. 58: 645–688.
8. HURLBERT, R. B., H. SCHMITZ, A. F. BRUMM, and V. R. POTTER. 1954. Nucleotide Metabolism. II. Chromatographic separation of acid-soluble nucleotides. Jour. Biol. Chem. 209: 23–39.
9. PABST LABORATORIES. 1956. Ultra-violet Absorption Spectra of 5'-Ribonucleotides. Circular OR-10, 21 pp. Pabst Brewing Co., Milwaukee, Wis.
10. ROWAN, K. S. 1957. Phosphorylated compounds in plants. I: Adenosine and uridine 5'-phosphates in pea seedlings. Jour. Exp. Bot. 8: 256–261.
11. SOLM, J. and W. Z. HASSID. 1957. Isolation of uridine diphosphate *N*-acetylglucosamine and uridine diphosphate glucuronic acid from mung bean seedlings. Jour. Biol. Chem. 228: No. 1, 357–364.

ROLE OF CALCIUM IN ABSORPTION OF MONOVALENT CATIONS[1, 2]

LOUIS JACOBSON, DAVID P. MOORE, AND RAYMOND J. HANNAPEL

DEPARTMENT OF SOILS AND PLANT NUTRITION, COLLEGE OF AGRICULTURE, UNIVERSITY OF CALIFORNIA, BERKELEY

The interaction of ions during the absorption process has frequently been exploited to study the mechanism and kinetics of ion absorption (4, 6, 17). Generally ions of similar valence have been used, although Middleton and Russell (14) have proposed a mechanism of ion absorption based primarily on the interaction of Sr and Rb. It has been recognized for some time that polyvalent cations often exert a striking influence on the absorption of monovalent ions. Viets (18) demonstrated that the alkaline earth metals and Al accelerated the accumulation of K and Br by excised barley roots and ascribed the effect to a metabolic or membrane phenomenon. Calcium had either a depressing or stimulating effect on K uptake depending upon the concentration of the latter (16).

Hydrogen ion has been implicated in the Ca effect. Arnon and Johnson (1) found that the deleterious effect of low pH on the growth of lettuce and tomato could be lessened by increasing the Ca content of the nutrient solution. More recently Ekdahl (5) has demonstrated that Ca effectively overcame the harmful influence of low pH on the elongation of root hairs. The work of Fawzy et al (7) indicated that pH was also a factor in the behavior of other polyvalent cations in addition to Ca. The stimulating effect increased markedly percentagewise as the pH of the external solution decreased. Wadleigh and Bower (19) were able to influence the relative Na and K contents of red kidney bean plants by varying the Ca content of the nutrient solution. Kahn and Hanson (12) suggested that Ca increased the affinity between K and a postulated carrier but decreased the velocity of the metabolic phase of K uptake in a second independent reaction.

In the experiments to be described, the effect of Ca on the absorption of several monovalent cations has been investigated over a wide range of pH. In addition, the relationships of various monovalent cations and pH in the absorption process have been examined.

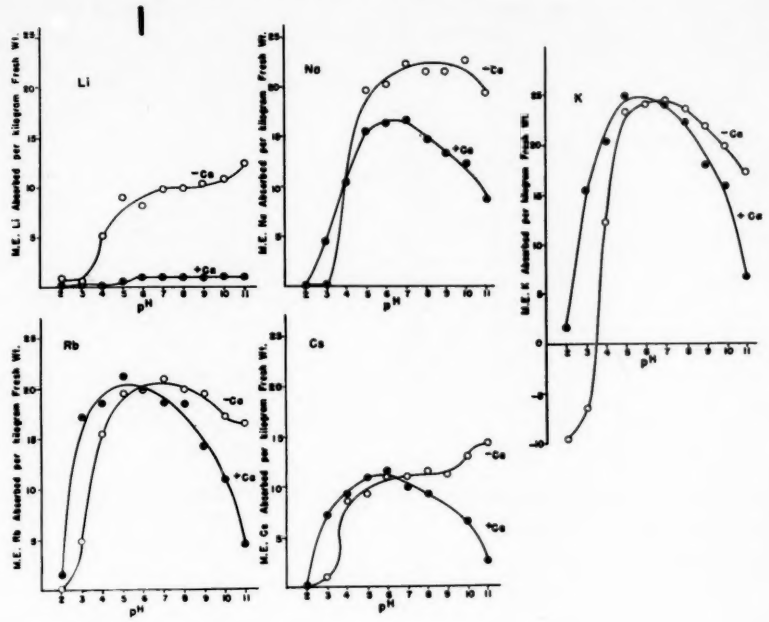
MATERIALS AND METHODS

Excised roots of barley, California Mariout 1956 crop, were used in these experiments. The plants were grown and the root material harvested as previously described (11). To minimize concentration changes in the solution, absorption experiments were carried out using 7 gm roots in 7 liters of solution. The solutions were aerated with CO_2 free air during the absorption period of 3 hours. In all experiments the pH was held constant during the course of the absorption period by appropriate additions of acid or base. These additions did not materially change the concentration of the solution. All absorption experiments were performed at 25° C. At the conclusion of an experiment, the roots were separated on a Nylon mesh filter and washed for 10 seconds with running distilled water. The washed roots were ignited and the various elements determined in an acidified solution of the ash. Li, Na, K, Rb, and Cs were determined by the flame photometric method.

Previous experiments had shown that after washing this material for 10 seconds, the non-metabolic or physical uptake corresponded to approximately 20% apparent free space (10). This correction was ap-

[1] Received August 10, 1959.

[2] This paper is based on work performed under contract No. At-(11-1)-34, Project 5, with the Atomic Energy Commission.



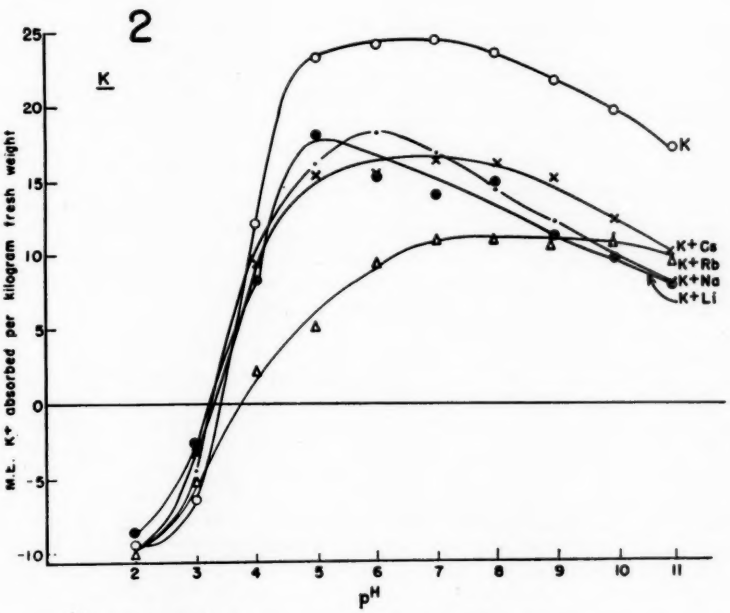


FIG. 1. Absorption of the alkali metal cations as a function of pH in the presence and absence of Ca. All salts present as the bromides at 5 meq/l each. Three hour absorption period.

FIG. 2. Effect of alkali metal cations on the absorption of K as a function of pH. All salts present as the bromides at 5 meq/l each. Three hour absorption period.

plied to all data. In most cases, the correction had relatively little effect on the shape or magnitude of the absorption curves.

Results

In the first experiment, the effect of Ca on the absorption of the alkali metal cations was determined over a broad pH range. The concentration of the solutions was 5 meq/l with respect to the particular cation being studied. The solutions were adjusted to pH values ranging from 2 to 11 by adding HBr to the hydroxide solution of the cation. By this procedure all solutions had identical alkali metal cation concentrations. For each of the elements, the absorption in the absence and presence of added Ca was determined. In addition to the test cation the Ca series contained 5 meq/l of Ca as $CaBr_2$ otherwise these solutions were prepared similarly to the single salt solutions. It is recognized that the extremes of the pH range studied are not ordinarily considered physiological. During short term experiments, however, the absorption mechanism is still operative at these extremes.

The data for the five elements are presented in figure 1. For K the stimulating effect of Ca is present at pH values below about 6.5. The percentage stimulation increased markedly with decreasing pH as observed by Fawzy et al (7). Above pH 6.5 Ca exerts a depressing effect on K absorption; the effect increases with pH. At pH 11 the absorption is reduced by 60 % when Ca is present. The qualitative effect of Ca on Rb and Cs absorption is similar to that on K although the absorption pattern for Cs in the absence of Ca is different from that of K and Rb. Over most of the pH range the absorption rate of Cs is about half that of K or Rb and at high pH values Cs absorption increases appreciably whereas K and Rb absorption decreases.

This root material absorbs Na almost as rapidly as it does K, but the effect of Ca on the two is quite different. In contrast to its effect on K absorption, Ca exerts a marked depressing effect on Na absorption over most of the pH range. Only at pH values below 4 does the presence of Ca show a stimulating effect and even then it is relatively small.

Of the five alkali metal cations studied in single salt solutions, Li is absorbed the least. Slightly less Li is absorbed than Cs, otherwise the two absorption curves are similar. Ca has a very striking effect on Li absorption, the presence of Ca almost completely inhibiting the absorption of Li over the entire pH range.

In figure 2 data for the effect of Li, Na, Rb, and Cs on the absorption of K at various pH values are given. The solutions contained 5 meq/l each of K and the test cation. The anion was Br and the pH adjusted and controlled as previously described. All four of the test cations significantly reduced K absorption: Li, Na, and Cs to approximately the same degree and Rb to a greater extent at the lower pH values. Other data showed the depressing effect to be mutual, i.e., the absorption of the test cation also was decreased in the presence of K. The necessity of relating ion competition to pH is clearly illustrated. At pH 5, Rb decreases the absorption of K by about 80 %. At pH 7 the decrease at the same concentration of Rb is about 55 %.

Table I

Retention of Potassium by Barley Roots as Function of pH in Presence and Absence of Calcium

pH*	K Content** (meq/kg fresh weight)	
	+ Ca***	− Ca
5	13.6	13.6
4	13.0	8.53
3	10.3	2.67
2	2.67	0.83

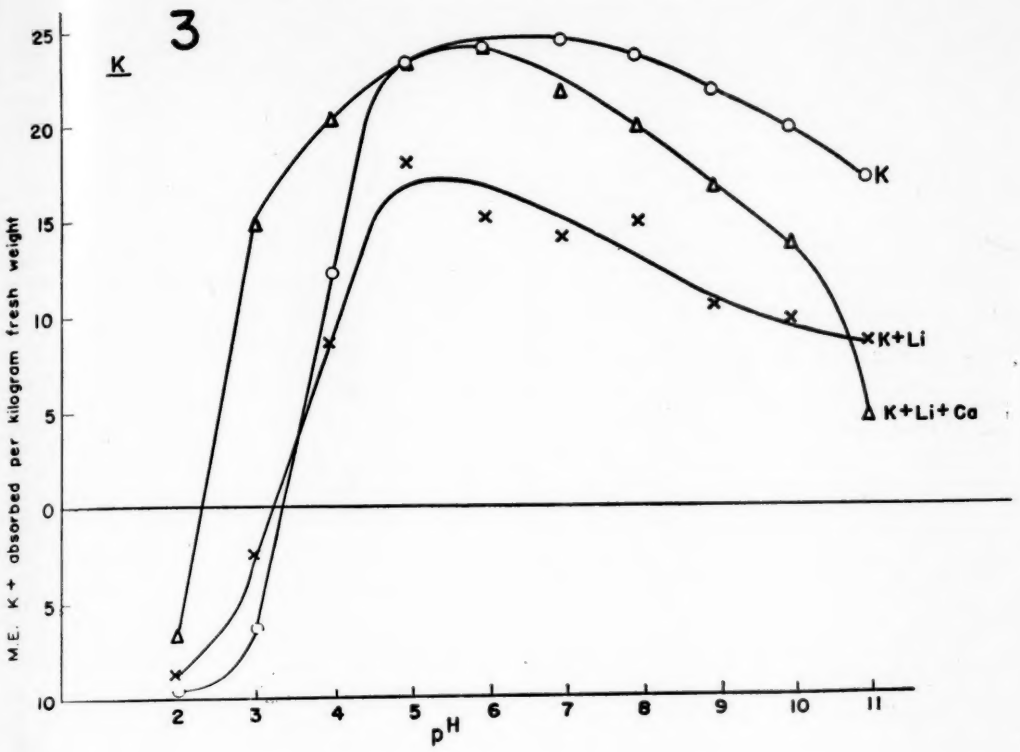
* pH adjusted and maintained with HBr for duration of experiment, i.e., 3 hours.
** Initial K content = 13.6 meq/kg fresh weight.
*** Concentration of added $CaBr_2$ = 5 meq/l.

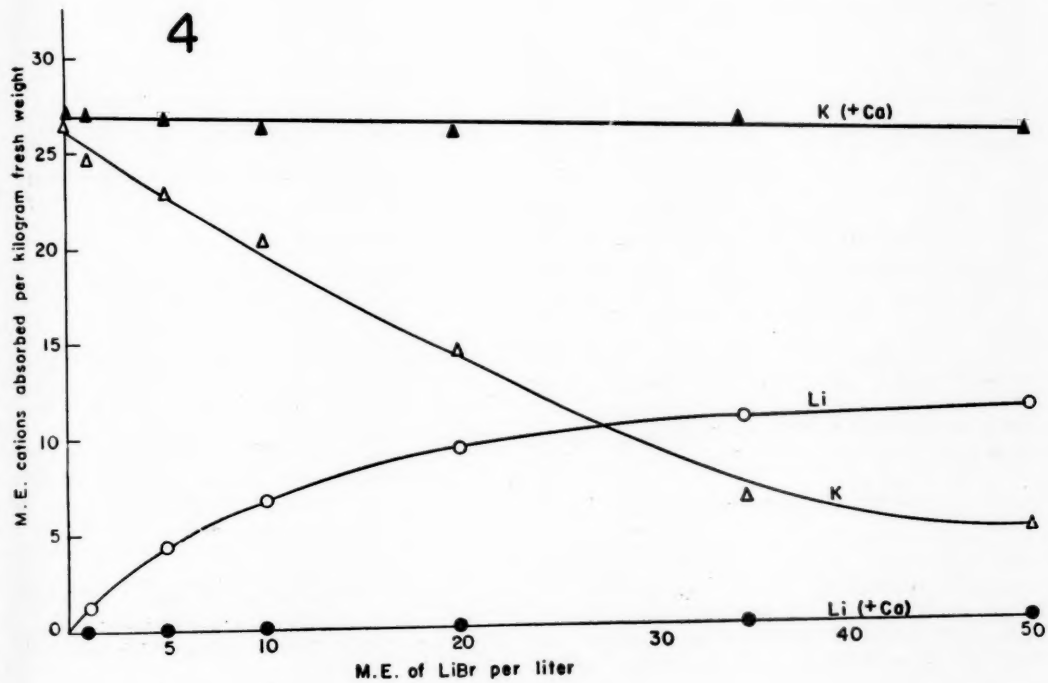
Not only is Ca effective in enhancing the absorption of K, but it is equally effective in decreasing the loss of previously absorbed K when roots are subjected to low pH. The data in table I were obtained from an experiment in which roots without prior treatment were placed for 3 hours in solutions at different pH values. One series consisted of distilled water adjusted to various pH values with HBr. The other series contained 5 meq/l of Ca as $CaBr_2$ in addition. The initial K content of the roots was 13.6 meq/Kg fresh weight. At pH 5 no K was lost in either series. As the pH decreased considerably greater losses of K took place in the minus Ca series as compared to the plus Ca series.

The interrelationship between K, Li, and Ca was examined more thoroughly. The absorption of K from solutions of KBr, KBr + LiBr, and KBr + LiBr + $CaBr_2$, all at 5 meq/l was determined as a function of PH. The data for the three solutions are summarized in figure 3. As shown above, Li represses the absorption of K but the addition of Ca to the K-Li system increases the K absorption to a level comparable to that obtained from a K-Ca system. At no pH value was there any significant uptake of Li from the K-Li-Ca system. Apparently Ca, in mixtures of K and Li, exerts independent effects upon the

Fig. 3. Absorption of K as influenced by Li and Li + Ca at various pH values. All salts present as bromides at 5 meq/l each. Three hour absorption period.

Fig. 4. Absorption of K from 5 meq/l KBr in the presence of increasing amounts of LiBr with and without the addition of 5 meq/l of $CaBr_2$. Three hour absorption period, pH 6.5.


3
K
M.E. K+ absorbed per kilogram fresh weight
pH
K
K+Li
K+Li+Ca


4
M.E. cations absorbed per kilogram fresh weight
M.E. of LiBr per liter
K (+Ca)
Li
K
Li (+Ca)

two ions. Just as in single salt solutions, K absorption is stimulated at the lower pH values and Li absorption is abolished in the presence of Ca.

The absorption of K in the presence and absence of Ca from solutions containing various ratios of K to Li was compared. One set of solutions contained 5 meq/l of KBr and increasing amounts of LiBr up to 50 meq/l. The other set was similar but contained 5 meq/l of Ca as $CaBr_2$. In both sets the pH was controlled at 6.5 and the absorption period was 3 hours. In this particular root material and at these concentrations, Ca has a negligible effect on the absorption of K at pH 6.5. The results are given in figure 4. In mixtures of K and Li, the absorption of K is reduced with increasing concentration of Li. At 50 meq/l of Li, K absorption falls to about 20 % of its absorption in the absence of Li. When Ca is present Li essentially exerts no effect even at Li-K ratios of 10 : 1.

Hydrogen is present on root surfaces in an exchangeable form (20). Burström (3) related the rate of hydrolysis of sucrose by wheat roots to the H adsorbed on the root surfaces. He varied both the hydrolysis and the adsorbed H with equimolar mixtures of NaCl and $CaSO_4$. Since there appeared to be a strong relation between Ca and H, an attempt was made to correlate the stimulating effect of Ca and other polyvalent cations with their ability to compete with H on the root surface. A relatively high root to solution ratio of 20 gm roots to 100 ml solution was used. The pH of the solutions of various bromide salts was adjusted to 4.00. Roots with no treatment other than the usual preparatory washing were placed in the solution and the pH measured. To avoid complications induced by metabolic absorption only the pH values at the end of 2 minutes were considered. Contact between the roots and the electrodes was avoided by shielding the latter with a stainless steel screen suspended about 1 cm from the electrodes. The pH values obtained are listed in table II. Although 2 minutes may not have been sufficient time for equilibration, the polyvalent cation treatments did result in lower pH values indicating that these ions were more effective in preventing H sorption than the monovalent cations. In other experiments at higher pH values evidence was obtained suggesting that the untreated roots contain exchangeable H and the polyvalent cations displace more of this H than monovalent cations. In a typical case roots were placed in 0.005 *N* KBr at pH 5.25. In 2 minutes the pH fell to 4.78. When placed in 0.005 *N* $CaBr_2$ the pH dropped to 4.49 in the same time period. In the absence of salts, the pH remained constant at 5.25.

Discussion

The absorption of cations by barley roots cannot be dissociated from hydrogen ion effects. Injury and competition with H and other cations are readily observed (8, 11, 15). The conclusion of Sutcliffe (17) that absorption is insensitive to external pH is not tenable in view of the results reported here and the above cited references. The effect of pH on the absorption of the alkali metal cations is similar qualitatively but differs quantitatively. These differences in specific behavior appear to be involved when absorption from mixtures of monovalent cations occur. At equimolar concentrations of K and an accompanying monovalent cation the ratio of K absorbed from KBr solution to K absorbed from the mixture varies with pH. This may be quite pronounced in some cases and emphasizes the importance of rigorous pH control during competitive experiments. Quantitative relationships between two competing cations may be determined largely by the pH of the solution. With the material used here there is little interference with K absorption at pH 3.5 or 4 by the added monovalent cations with the exception of Rb. At pH 5 or higher all of the added cations cause a marked depression of K absorption. An examination of figure 2 indicates that in order to draw meaningful conclusions from mixtures of two salts it is necessary to pay considerable attention to pH relationships because of the three way interaction which exists involving H and the two cations.

The relationship between pH and absorption is particularly striking in mixtures of mono and polyvalent cations. The effect of Ca on the absorption of the monovalent cation is both a function of the pH of the external solution and the particular monovalent cation involved. In solutions of K, Rb, and Cs, the presence of Ca may enhance, depress, or have no effect on the absorption of the alkali metal cation depending upon the pH of the solution. The absorption of Na is strongly depressed by Ca over much of the pH range studied. The effect of Ca on Li absorption is

Table II

Effect of Barley Roots on pH of Various Salt Solutions Initially Adjusted to pH 4.00

Added salt*	pH**
None	4.79
LiBr	4.71
NaBr	4.72
NH_4Br	4.71
KBr	4.69
RbBr	4.70
CsBr	4.74
$BeBr_2$	4.30
$MgBr_2$	4.65
$CaBr_2$	4.59
$SrBr_2$	4.64
$BaBr_2$	4.60
$AlBr_3$	4.00
$LaBr_3$	4.31
$ThBr_4$	4.28

* 1 meq/l of indicated salt added to solution of 1 meq/l KBr.

** pH measured 2 minutes after addition of 20 g roots to 100 ml solution. Electrodes separated from roots by wire mesh.

unique in that the presence of Ca almost completely inhibits the absorption over the entire pH range.

These differences in the effect of Ca on the absorption of the various alkali metal cations cannot be correlated with relative rates of absorption of the cations from single salt solutions. The three ions Na, K, and Rb are absorbed at approximately the same rate by the barley root material yet Ca affects the absorption of Na quite differently than it does that of K and Rb. The absorption rates of Cs and Li are similar in the absence of Ca but react very differently to the presence of Ca. These observations and the fact that the Ca effect is a function of pH indicate that a valid explanation must depend upon the interaction of Ca, H, and the specific monovalent cation being absorbed.

If the absorption from mixtures of K and Li is examined, it is observed that there is a mutual influence of one cation upon the other. When the K concentration is maintained constant and the Li concentration is increased, the absorption of K decreases considerably and the Li absorption increases. The presence of Ca completely destroys this relationship: K absorption proceeds as if there were no Li present, and Li absorption is abolished. In order to minimize the complications resulting from the Ca-H interaction with K absorption, this experiment was carried out at pH 6.5 where Ca has little effect upon the absorption of K from KBr solutions with this material. However, the data show that at any pH the addition of Ca to mixtures of K and Li results in absorption approximating that obtained from mixtures of K and Ca at the same pH. It seems reasonable to assume that Li interferes with K absorption at the absorption reaction site and that the action of Ca is to prevent access of Li to this step. It is interesting that this behavior is not confined to barley since an almost identical K-Li-Ca interrelationship was observed with excised pea roots grown and prepared in a similar fashion as the barley roots. Preliminary data also indicated that the partial blocking of Na absorption by Ca results in an increased absorption of K from mixtures of Na and K.

The effect of Ca on the absorption of K, Rb, Cs, and to a lesser extent on Na, at low pH values may be considered in an analogous manner. The absorption of K decreases as the concentration of either H or Li in the solution increases. In both cases Ca is effective in overcoming the depressing effect of the interfering ions to a considerable extent. Roots are known to take up H in an exchangeable form. As might be expected, the higher the valence of the added cation, the more effectively the uptake of H is blocked and the more readily it is displaced. The charge is more important than the species of ion. This is similar to the effect of polyvalent cations on the absorption of K at low pH values (7) and may explain the lack of specificity in stimulation. Since the changes in H concentration occurred within 2 minutes, the site of the exchange reaction is most likely at or near the cell surface. Because of possible changes in the chemical potential of ions adsorbed on surfaces of cells (13), it does not appear feasible to interpret the results quantitatively.

When untreated roots are placed in dilute acid solution irreversible injury occurs and there is a leakage of cell contents (11). The presence of Ca in the external solution greatly lessens the leakage of K from the roots again suggesting that Ca decreases the uptake of H.

The absorption behavior of an anion may have an appreciable effect on the absorption rate of the accompanying cation (9). The change in Br concentration of the external solution resulting from the addition of $CaBr_2$ might therefore have influenced the absorption of the cation. If such an effect does exist here, however, it is small compared to the Ca effect. Increasing the Br concentration several fold (as Li Br) in the presence of Ca had essentially no effect on the rate of K absorption. Furthermore a similar effect has been observed even when salts of a poorly absorbed anion were used, i.e., SO_4 salts.

The data of figure 4 can neither be explained on the basis of relative binding affinities of Li, K, and Ca to an absorption site or carrier, nor on the basis of multiple absorption sites. At pH 6.5 Ca almost completely represses Li absorption but has little or no effect on K absorption in the presence or absence of Li, yet Li markedly depresses K absorption in the absence of Ca at this pH. In order to reconcile these observations, it is postulated that the effect of Ca is at some point other than the actual absorption site, possibly at or near the cell surface. Ca is known to alter the properties of cell surfaces. It has been shown, for example, that Ca decreases the charge on root surfaces (2). For an ion to be absorbed it must first cross the barrier imposed by the presence of Ca and then react at the absorption site. The Ca modified surface acts as an efficient barrier for H and Li, less so for Na, and least if at all for K and presumably Rb and Cs. In such a system the interaction between two monovalent cations in the absorption process would be a function of the selectivity of the barrier and the relative rates of absorption of the cations reaching the absorption site. It would thus appear that the stimulation of K absorption by Ca is in reality a blocking of an interfering cation such as H or Li.

Summary

Hydrogen ion has been shown to influence the absorption of monovalent cations in single salt solutions. This influence is reflected in the absorption behavior from mixtures of the monovalent cations and is instrumental, in part, in determining the relative absorption rates. The effect of Ca on absorption is likewise related to pH as well as the specific monovalent cation. The absorption of K, Rb, and Cs is enhanced at low pH by Ca; Na only slightly. Li absorption is repressed almost completely by Ca at all pH values. In mixtures of Li and K the presence of Ca very effectively blocks Li absorption and eliminates the Li interference with the absorption of K. The H-Ca interaction is similar to the Li-Ca inter-

action and accounts for the stimulating effect at low pH. A correlation exists between the charge of a cation and its effectiveness in displacing H on the root surface.

To explain the interrelationships in the Li-K-Ca system or the H-K-Ca system, it is postulated that the presence of Ca in the solution creates a barrier probably at the cell surface. This barrier which is particularly effective in blocking Li and H determines the relative amounts of the cations reaching the absorption site. The stimulating effect of Ca is considered to be essentially a blocking of interfering ions.

Acknowledgements

The writers are indebted to Mr. C. K. Stromberg for preliminary work on the Li-Ca system and to Mr. H. M. King for technical assistance throughout this study.

Literature Cited

1. Arnon, D. I. and C. M. Johnson. 1942. Influence of hydrogen ion concentration on the growth of higher plants under controlled conditions. Plant Physiol. 17: 525–539.
2. Briggs, G. E. and A. B. Hope. 1958. Electric potential differences and the Donnan equilibrium in plant tissues. Jour. Exp. Bot. 9: 365–371.
3. Burström, Hans. 1941. Studies on the carbohydrate nutrition of roots. Lantbrukshögskol. Ann. 9: 264–284.
4. Collander, R. 1941. Selective absorption of cations by higher plants. Plant Physiol. 16: 691–720.
5. Ekdahl, Ivar. 1957. The growth of root hairs and roots in nutrient media and bidistilled water, and the effects of oxalate. K. Lantbrukshögskol. Ann. 23: 497–518.
6. Epstein, E. and C. E. Hagen. 1952. A kinetic study of the absorption of alkali cations by barley roots. Plant Physiol. 27: 457–474.
7. Fawzy, H., R. Overstreet, and L. Jacobson. 1954. The influence of hydrogen ion concentration on cation absorption by barley roots. Plant Physiol. 29: 234–237.
8. Fried, Maurice and J. C. Noggle. 1958. Multiple site uptake of individual cations by roots as affected by hydrogen ion. Plant Physiol. 33: 139–144.
9. Hoagland, D. R. 1948. Inorganic plant nutrition. Chronica Botanica Co., Waltham, Mass.
10. Jacobson, L., R. J. Hannapel, and D. F. Moore. 1958. Non-metabolic uptake of ions by barley roots. Plant Physiol. 33: 278–282.
11. Jacobson, L., R. Overstreet, H. M. King, and R. Handley. 1950. A study of potassium absorption by barley roots. Plant Physiol. 25: 639–647.
12. Kahn, J. S. and J. B. Hanson. 1957. The effect of calcium on potassium accumulation in corn and soybean roots. Plant Physiol. 32: 312–316.
13. McLaren, A. D. 1957. Concerning the pH dependence of enzyme reactions on cells, particulates, and in solution. Science 125: 697.
14. Middleton, L. F. and R. Scott Russell. 1958. The interaction of cations in absorption by plant tissues. Jour. Exp. Bot. 9: 115–127.
15. Nielsen, T. R. and R. Overstreet. 1955. A study of the role of hydrogen ion in the mechanism of potassium absorption by excised barley roots. Plant Physiol. 30: 303–309.
16. Overstreet, R., L. Jacobson, and R. Handley. 1952. The effect of calcium on the absorption of potassium by barley roots. Plant Physiol. 27: 583–590.
17. Sutcliffe, J. E. 1957. The selective uptake of alkali cations by red beet tissue. Jour. Exp. Bot. 8: 36–49.
18. Viets, Frank G. 1944. Calcium and other polyvalent cations as accelerators of ion accumulation by excised barley roots. Plant Physiol. 19: 466–480.
19. Wadleigh, C. H. and C. A. Bower. 1950. The influence of calcium ion activity in water cultures on the intake of cations by bean plants. Plant Physiol. 25: 1–12.
20. Williams, D. E. and N. T. Coleman. 1950. Cation exchange properties of plant root surfaces. Plant and Soil II: 243–256.

EFFECT OF GIBBERELLIC ACID AND PHOTOPERIOD ON INDOLEACETIC ACID OXIDASE IN LUPINUS ALBUS L. [1, 2]

RONALD WATANABE [3] AND ROBERT E. STUTZ [4]

DIVISION OF BIOLOGICAL AND MEDICAL RESEARCH, ARGONNE NATIONAL LABORATORY, LEMONT, ILLINOIS

Recent reports from several laboratories (1, 6, 8, 12) on the effect of gibberellin treatment upon indole-3-acetic acid (IAA) oxidase activity in plant tissues appear to present controversial results. Kato and Katsumi (6) and Brian and Hemming (1) have reported that gibberellin treatment of either pea seedlings or pea internode sections did not affect the IAA oxidase activity in the plant tissues. On the other hand, Pilet (8) has reported that the IAA oxidase activity was reduced in excised carrot tissues which were cultured in vitro with low concentrations of gibberellins.

In this paper we have investigated the growth stimulation and IAA oxidase activity in intact lupine plants which had been treated over an extended period with low doses of gibberellic acid (GA) and cultured under two different daylengths. A preliminary report of this work has been presented (12). The results support the views proposed by Vlitos and Meudt (15) and Galston and Warburg (3) that growth stimulation by GA is probably mediated through an auxin-sparing mechanism. Recently Philips and coworkers (7) have presented evidence that GA does not function by an auxin-sparing mechanism but forms a growth-stimulating complex which acts both as an auxin and gibberellin-like compound. It is suggested that had the other investigators (1, 6) assayed tissues which had been treated with GA over a longer period of time that they may have observed a reduction in IAA oxidase activity.

MATERIALS AND METHODS

Lupine seeds (*Lupinus albus* L.) were sown in flats with soil, and watered adequately with tap water. When the seedlings were 14 days old, they were transplanted in soil into 4 in clay pots. Approximately 100 seedlings were placed under each of two photoperiods, 8 hour and 16 hour, for the duration of the experiment. The normal daylength was extended by supplemental incandescent lights for the 16 hour photoperiod. When the plants were 30 days old, 60 plants of uniform size were selected from each photoperiod group. One-half of each lot was treated with GA by applying 5 μl of 0.001 % aqueous-Tween 20 solution containing 1 mg GA per ml to the terminal bud of each plant. Control plants were treated with 0.001 % aqueous-Tween 20 solution without GA. This 5 μg GA application was repeated at 2 to 3 day intervals until a total of 50 μg had been applied. The GA was supplied by Dr. Curt Leben, Eli Lilly and Company, Indianapolis, Ind. Ten days after the final treatment, all plants were harvested and measured individually for main-axis length and for number of nodes. They were then sectioned into stems, hypocotyls, buds, and leaves; frozen in liquid nitrogen and maintained at $-20°$ C until enzyme preparations were made.

The preparation of crude enzyme extracts and manometric assay procedures are those described by Stutz (10). The crude enzyme extract was prepared by grinding the tissues in a chilled mortar with sand and 1 ml water per gram fresh weight of tissue. The homogenate was squeezed between double layers of cheesecloth. The filtrate was centrifuged at 20,000 $\times$ G for 15 minutes at 0° C to give a clear supernatant solution. If not used immediately, the supernatant was stored in a frozen state.

The IAA oxidase activity was determined by conventional Warburg manometry (14). Each flask contained 1 ml enzyme extract, 1 ml 0.4 M pH 6.3 phosphate buffer, 0.5 ml 0.6 M IAA solution, and 0.5 ml water. The center well contained 0.1 ml of 1 *N* KOH solution and a paper wick. The 0.6 M IAA stock solution was prepared by adding sufficient dilute KOH solution to dissolve the IAA and to adjust the final pH to 7.0. The solution was kept in the refrigerator when not in use.

RESULTS AND DISCUSSION

Table I shows a typical net increase in main axis length due to photoperiod and treatment with GA. Plants treated with GA had a significant increase in growth of the main axis and in the number of nodes under both photoperiods. Height of the main axis increased with increased duration of photoperiod, but the rate of organ differentiation was not affected. This photoperiodic effect had been observed earlier (11). It is evident from table I that the increased stem elongation caused by both GA and long photoperiod were to a large extent additive.

The effect of GA and photoperiod on IAA oxidase activity in the buds and stems is shown in figures 1

[1] Revised manuscript received August 14, 1959.

[2] This work was performed under the auspices of the U. S. Atomic Energy Commission.

[3] Present address: Department of Chemistry, University of Oklahoma, Norman.

[4] Present address: Research Laboratory of the Western Pine Association, 7733 SE 13th Avenue, Portland 2, Ore.

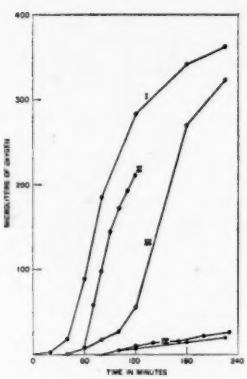


FIG. 1 (*left*). Oxygen uptake by IAA oxidase from cell free extracts of bud tissues. Curve I, 16 hour photoperiod. Curve II, dilution of IAA oxidase preparation from GA treated plants with equal volume of water. Curve III, 8 hour photoperiod. Curve IV, 8 hour and 16 hour photoperiod with GA treatment.

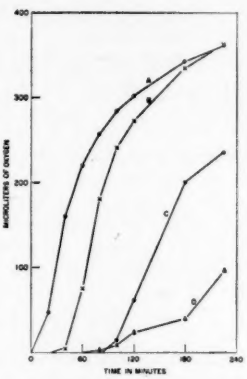


FIG. 2 (*right*). Oxygen uptake by IAA oxidase from cell free extracts of stem tissues. Curve A, 8 hour photoperiod. Curve B, 8 hour photoperiod and GA treated. Curve C, 16 hour photoperiod. Curve D, 16 hour photoperiod and GA treated.

and 2, respectively. The curves are representative data from three experiments. The total O_2 uptake varied between experiments, but the relative position of each curve with respect to each other was essentially the same. The IAA oxidase activity of bud preparations was strikingly inhibited by GA under both photoperiods (fig 1, curve IV). GA effect on the oxidase activity from stem tissues was not significant with the 8 hour photoperiod (fig 2). With the longer photoperiod, the stem oxidase activity was significantly reduced by GA treatment. Moreover, increased photoperiod also reduced the oxidase activity appreciably. This photoperiodic effect on IAA oxidase activity from stems was noted previously (11).

Addition of GA to either crude or electrophoretically homogenous enzyme preparations from lupine plants resulted in no change in enzyme activity (R. E. Stutz, unpublished). Similar results were observed by others, (1, 6). There was slight enzyme activity in extracts from leaves and hypocotyls. Neither GA treatment nor photoperiod affected the IAA oxidase activity in these tissues.

In order to explain a possible mechanism for GA stimulation of growth, several investigators (1, 3, 15) have proposed an auxin-sparing mechanism in which a third factor, presumably an IAA oxidase inhibitor of phenolic nature, is involved. These views are supported by the work reported here, in that dilution of representative enzyme extract from GA treated bud tissues increased the IAA oxidase activity (fig 1, curve II), by supposedly reducing the level of an inhibitor, which was reported by Tang and Bonner (13). Galston (2, 4) has reported that an inhibitor to auxin destruction actually increases in GA treated tissues. It is also known by the work of Hillman and Galston (5) that IAA oxidase inhibitor level is controlled by the red-far-red mechanism. If a similar type of photocontrol of IAA oxidase inhibitor concentration occurs in lupine plants, it could explain the greater elongation of stem axis of plants grown on a 16 hour photoperiod as compared to those on 8 hour photoperiod.

Without a more direct method for determining the changes in both auxin and inhibitor levels due to GA and photoperiod treatments, it can only be speculated that these external factors stimulate growth by an auxin-sparing mechanism. Although gibberellins have not been demonstrated to be present in vegetative lupine tissues, the use of GA for the study of its stimulation of growth in lupine is not without significance since gibberellin-like compounds have been found in green lupine seeds by Phinney and coworkers (9).

SUMMARY

I. Application of 50 μg GA to terminal buds of lupine plants in 5 μg aliquots over a period of 31 days, resulted in a decrease in IAA oxidase activity in bud and stem tissues but had no significant effect in leaves and hypocotyls. This treatment increased the length of the main axis and the number of visible nodes.

II. Plants cultured under 16 hour photoperiod increased the stem length significantly over those on 8 hour photoperiod. The increased photoperiod reduced the IAA oxidase activity in stem tissues but had opposite effect in bud tissue. Photoperiod did not affect activity in leaves and hypocotyls.

III. When GA and 16-hour photoperiod are ap-

TABLE I

EFFECT OF PHOTOPERIOD AND GIBBERELLIC ACID ON GROWTH OF LUPINUS ALBUS L.*

	PHOTOPERIOD 8 HRS		PHOTOPERIOD 16 HRS	
	CONTROL	GA TREATED	CONTROL	GA TREATED
Growth of stem (cm)**	11.8±0.5†	21.2±0.6	22.9±1.0	30.7±1.2
Number of nodes***	13.9±0.2	17.1±0.4	14.7±0.4	17.6±0.5

Each datum represents the average from 28 to 30 plants, 71 days after planting and 41 days after initial treatment.
** Differences between treated and control and between photoperiod treatments are significant at the 1 % level.
*** Differences between treated and control are significant at the 1 % level.
† S.E. of the means.

plied concurrently, the stimulation of growth of main axis is additive. Also, the depression of IAA oxidase activity is additive when the two external factors are applied jointly.

IV. These results are in agreement with the three-factor system in which GA has an auxin-sparing action by regulating the concentration of a natural growth inhibitor.

Acknowledgements

The authors wish to thank Dr. Norbert Scully, Dr. John Skok, and Dr. Fritz Schlenk for their encouragement and advice throughout this work and they wish to thank Mr. George Kostal and Mrs. Sarah Berliner for technical assistance.

Literature Cited

1. Brian, P. W. and H. G. Hemming. 1958. Complementary action of gibberellic acid and auxins in pea internode extension. Ann. Bot. (N.S.) 22: 1–17.
2. Galston, A. W., Oct. 1957. Investigations on a substance whose level in tissue is controlled by the red-far-red reversible photoreaction. In: Photoperiodism and Related Phenomena in Plants and Animals. An international symposium held at Gatlinburg, Tenn.
3. Galston, A. W. and H. Warburg. 1959. An analysis of auxin-gibberellin interaction in pea stem tissue. Plant Physiol. 34: 16–22.
4. Galston, A. W. 1957. Studies on indoleacetic acid oxidase and its inhibitor in light-grown peas. Plant Physiol. 32 suppl.: xxi.
5. Hillman, W. A. and A. W. Galston. 1957. Inductive control of indoleacetic acid oxidase activity by red and near infrared light. Plant Physiol. 32: 129–135.
6. Kato, J. and M. Katsumi. 1958. Effect of gibberellins on IAA oxidase. Naturwiss. 45: 344.
7. Phillips, I. D. J., A. J. Vlitos, and H. Cutler. 1959. The influence of gibberellic acid upon the endogenous growth substances of the Alaska pea. Contrib. Boyce Thompson Inst. 20: 111–120.
8. Pilet, P. E. 1957. Action des gibberellines sur l'activité auxin-oxydasique de tissus cultivé in vitro. Compt. rend. acad. sci. (France) 245: 1327–1328.
9. Phinney, B. O., C. A. West, M. Ritzel, and P. M. Neely. 1957. Evidence for "gibberellin-like" substances from flowering plants. Proc. Natl. Acad. Sci., U.S. 43: 398–404.
10. Stutz, R. E. 1957. The indole-3-acetic acid oxidase of *Lupinus albus* L. Plant Physiol. 32: 31–39.
11. Stutz, R. E. 1955. Indoleacetic acid oxidase and photoperiod. Argonne Natl. Lab., Biol. and Med. Research Div., Quarterly Report. ANL-5486: 59–60.
12. Stutz, R. E. and R. Watanabe. 1957. The gibberellins. II. The effect of gibberellic acid and photoperiod on indoleacetic acid oxidase in *Lupinus albus* L. Argonne Natl. Lab., Biol. and Med. Research Div., Semiannual Report. ANL-5732: 107–109.
13. Tang, Y. W. and J. Bonner. 1948. The enzymatic inactivation of indole acetic acid. II. The physiology of the enzyme. Amer. Jour. Bot. 35: 570–578.
14. Umbreit, W. W., R. H. Burris, and J. F. Stauffer. 1957. Manometric Techniques and Tissue Metabolism. Burgess, Minneapolis.
15. Vlitos, A. J. and W. Meudt. 1957. Relationship between shoot apex and effect of gibberellic acid on elongation of pea stems. Nature 180: 284.

DIFFERENTIAL ABSORPTION OF METAL CHELATE COMPONENTS BY PLANT ROOTS [1]

LEE O. TIFFIN, JOHN C. BROWN, AND ROBERT W. KRAUSS

MINERAL NUTRITION LABORATORY, AGRICULTURAL RESEARCH SERVICE, U. S. DEPARTMENT OF AGRICULTURE, BELTSVILLE, MARYLAND, AND DEPARTMENT OF BOTANY, UNIVERSITY OF MARYLAND, COLLEGE PARK

The use of synthetic metal chelators in bionutrition has become well known since the initial work of Schatz and Hutner (8) and Hutner, et al (3). Early investigators (1, 3, 8, 12,13) considered the chelating agent as a carrier which delivered metals to absorbing surfaces, but was not itself absorbed. This view was soon modified, however, when the uptake of the chelating agent or a decomposition product was demonstrated (2, 4, 6, 11) by use of C^{14}-tagged chelates. The increased concentrations of iron in plant materials (2, 4, 6, 11, 14), in addition to the presence of C^{14}, suggested the probability that metal chelate components were absorbed together. By use of N^{15}-tagged chelate, Wallace and co-workers (15) give data from citrus which indicate an equivalent uptake of iron and chelating agent. In later work (16), however, they reported cases in which high ratios of iron to chelating agent indicated that the uptake of these components may not be equivalent. The same lack of equivalence was shown by Krauss and Specht (4) in studies with green algae. Although some evidence may be adduced in favor of equivalent uptake, it is possible that considerable exchange may also take place at the root surface.

This paper reports the differences in the quantities of iron and of chelating agent absorbed from an iron chelate by plant roots. The results indicate an absorption mechanism which involves some absorption of the chelating agent, but a much greater absorption of iron which was released from the chelate at the root surface.

MATERIALS AND METHODS

The plants were: Eldorado No. 882 zinnia (*Zinnia elegans* Jacq.), PI-54619-5-1 soybean (*Glycine max* (L.) Merr.), and Greystripe sunflower No. F-4688-20 (*Helianthus annuus* L.). The seed sources were: zinnia, F. W. Bolgiano & Co., Washington, D.C.; soybean, the Plant Industry Station, Beltsville, Md.; and sunflower, Northrup King & Co., Berkeley, Calif.

The chelating agent was ethylenediamine di(*o*-hydroxyphenylacetic acid)—an aminopolycarboxylic acid. This chelating agent is a phenolic analog of EDTA (5). It has been called EDDHA, APCA, EHPG, and Chel-138. In this paper the term EDDHA will be used. When combined with ferric iron this compound gives a deep red color. The absorption spectra of FeEDDHA at six concentrations are shown in figure 1. The optical density plotted against concentration follows Beer's Law up to 10 ppm Fe.

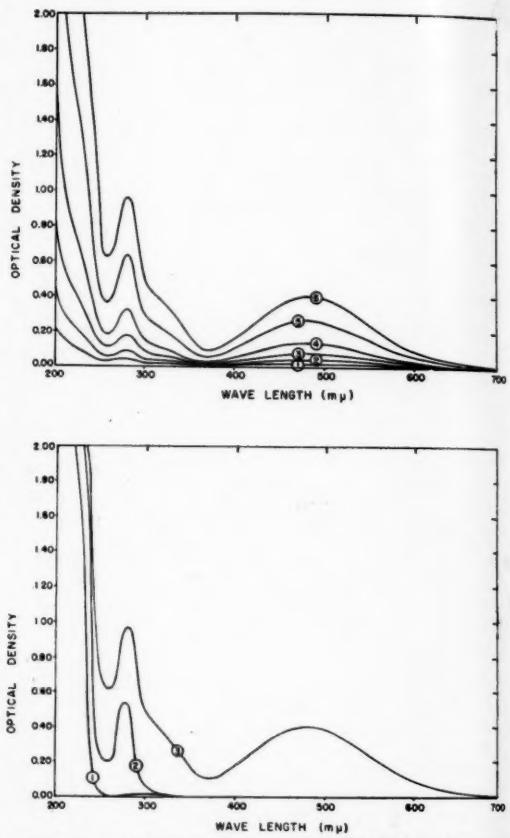


FIG. 1. Absorption spectra of FeEDDHA solutions. (Curves 1-6 correspond respectively to concentrations of 2, 4, 8, 16, 32, and 48 mg FeEDDHA per liter.)

FIG. 2. Absorption spectra of nutrient solutions. (Curves correspond to solutions as follows: 1. Nutrient solution alone, 2. Nutrient solution plus 50 mg EDDHA per liter, and 3. Nutrient solution plus 55 mg FeEDDHA per liter.)

[1] Received August 17, 1959.

The base nutrient medium was an iron-free modified Steinberg solution (10) adjusted to pH 6.8. A liter of the nutrient medium contained 150 mg $Ca(NO_3)_2 \cdot 4H_2O$; 35 mg $Mg(NO_3)_2 \cdot 6H_2O$; 9 mg NH_4NO_3; 12 mg K_2SO_4; 30 mg KNO_3; 5 mg K_2HPO_4; 1.3 mg $(NH_4)_2HPO_4$; 0.234 mg $MnCl_2 \cdot 4H_2O$; 0.204 mg H_3BO_3; 0.088 mg $ZnSO_4 \cdot 7H_2O$; 0.02 mg $CuSO_4 \cdot 5H_2O$, and 0.012 mg $Na_2MoO_4 \cdot 2H_2O$. This solution showed little absorbance at 280 mμ and no absorbance at 480 mμ when measured in a Beckman DU spectrophotometer (see curve 1, fig 2).

The nutrient plus EDDHA absorbs strongly at 280 mμ, but not at 480 mμ (see curve 2, fig 2). The Na and K chelates of EDDHA were found to absorb at 280 mμ. The nutrient medium plus FeEDDHA and other metal chelates of this acid absorb strongly at 280 mμ, but only FeEDDHA gives a specific but weaker absorbance at 480 mμ (see curve 3, fig 2). Due to the interfering absorbance of various compounds at 280 mμ, 480 mμ was the most satisfactory wavelength for the determination of FeEDDHA. Preliminary investigations disclosed that colored exudates could be obtained from stems of certain plants when their roots were in FeEDDHA. Later experiments with these exudates indicated an absence of interfering absorption by other compounds at 480 mμ. Thus the determination of FeEDDHA in both nutrient and exudate was possible.

Seeds of the test plants were germinated between layers of moist muslin on stainless steel wire frames placed in Pyrex trays. The muslin was kept moist by extending it over the edges of the wire frames into water maintained at one-fourth inch below the seeds. The seeds and trays were covered with aluminum foil and kept in darkness at 75° F for 3 days. Seedlings with roots one-half inch long were transferred to aerated nutrient solution and grown in a constant temperature room at 75° F. Illumination was provided for 16 hours each day by banks of fluorescent and incandescent lamps at an intensity of 1,500 ft-c. After the soybeans and sunflowers had grown for 3 days and the zinnias for 10 days, selected seedlings were bound in groups and transferred to individual jars of nutrient medium for further growth periods (12 additional days for soybeans and sunflowers and 20 days for zinnias). The number of seedlings in each group was as follows: four zinnia; ten soybean, and ten sunflower. In one experiment 30 sunflower plants were used.

For collection of exudate the roots of the plants were washed and the groups were placed individually in 1 liter beakers of aerated nutrient containing varied treatments of EDDHA and FeEDDHA. The beakers were covered with plastic in order to hold the plant stems in place and to prevent excessive evaporation of the solution. The stems were cut off 10 cm above the roots, bent over, and the cut ends were pushed through a hole in the plastic covering of a 50 ml beaker from which exudate samples could be pipetted for analysis (fig 3). Calibrated centrifuge tubes were used for collecting fractions at time intervals. Collecting assemblies were covered with bell jars and kept in darkness at 75° F.

Three methods were used for analyses of exudate and nutrient medium. The optical density of FeEDDHA was measured by a Beckman DU spectrophotometer. Total Fe was determined on ashed material by the colorimetric *o*-phenanthroline method (7). The iron chelating capacity of exudate or nutrient was determined as follows: 2.9 μg $Fe^{55, 59}$ with a specific activity of 937 cps/μg was added to each ml of exudate or nutrient. After adding radioiron the samples were brought to pH 6, heated to 90° C, then set aside to equilibrate for 12 hours. Samples were then brought to pH 8 and heated to 90° C to precipitate non-chelated iron, after which they were filtered through No. 42 Whatman paper into tin planchets, brought slowly to dryness, and counted in a proportional counter. For the nutrient and exudate controls no more than 1.5 and 0.5 %, respectively, of the added activity passed through the filter paper. By this method it was possible to identify very small quantities of iron which were taken as an index of the chelating capacity of nutrients or exudates.

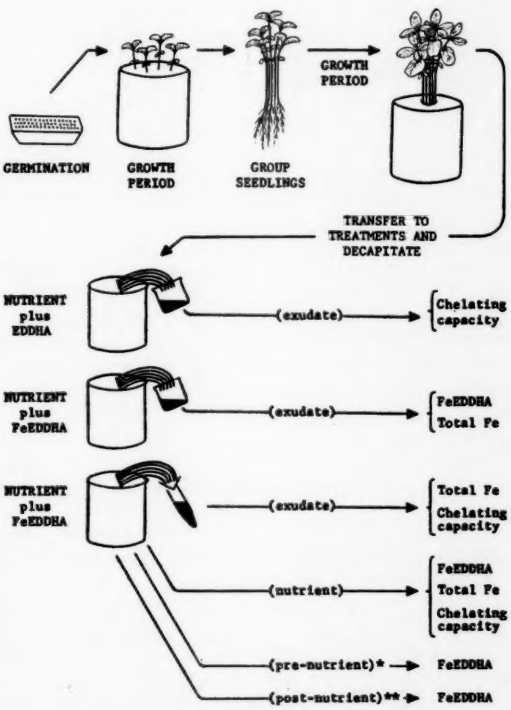


FIG. 3. Schematic representation of the procedure employed in treating and sampling plants and media to study the absorption of metal chelate components by plant roots.

TABLE I

EFFECT OF EDDHA IN NUTRIENT SOLUTION ON IRON-CHELATING CAPACITY OF EXUDATE FROM DECAPITATED ZINNIA, SUNFLOWER, AND SOYBEAN PLANTS

NUTRIENT SOLUTION TREATMENTS	CHELATING CAPACITY OF PLANT EXUDATES (IN TERMS OF $Fe^{55, 59}$ CHELATED)		
EDDHA PPM	ZINNIA PPM	SUNFLOWER PPM	SOYBEAN PPM
0	0.003	0.016	0.012
50	0.011	0.032	0.077
200	0.038	0.081	0.097
400	0.091	0.198	0.316
600	0.422	0.329	...

For determining chelating capacity of exudate, an aliquot was collected from groups of zinnia plants and groups of sunflower plants with roots in nutrient medium which contained 0, 50, 200, 400, and 600 mg EDDHA per liter. The same nutrient series was used in an experiment with soybeans except that the 600 ppm level was eliminated. Each of these experiments was repeated several times.

To determine in plant exudates the ratio of total iron to chelated iron, exudate was collected from additional groups of zinnia, soybean, and sunflower plants with roots in base nutrient containing 0, 55, 220, 440, and 660 mg FeEDDHA per liter.

In an experiment designed to give the time course of absorption, a base nutrient containing 55 mg FeEDDHA per liter was prepared and a 10 ml sample, hereafter designated "pre-nutrient," was set aside for later analysis. Thirty sunflower plants were then placed in 1 liter of the nutrient medium. After the pre-nutrient sample at zero time, a 5 ml sample of exudate and of nutrient was taken at 5, 10.5, 16.5, 23, 31.5, and 42.5 hours. Changes in FeEDDHA, total iron, and iron-chelating capacity of the nutrient solutions, and the concurrent changes in total iron and chelating capacity of the exudates were determined.

A final experiment was performed to determine whether the sunflower plants in the time-course experiment had absorbed iron and left EDDHA in the nutrient. The pre-nutrient sample taken at zero time and a sample of the nutrient after it had contained plants for 42.5 hours (hereafter designated "post-nutrient") were used for this purpose. The absorbance of these samples was first determined, after which excess Fe^{+++} was added to both the pre-nutrient and post-nutrient. Absorbance readings were then taken at time intervals to determine the relative rates and extent of the chelation of Fe^{+++} as FeEDDHA.

RESULTS

The effect of EDDHA in the iron-free nutrient medium on the iron chelating capacity of plant exudates is given in table I. For the exudates of all three plant species there is an upward trend in iron chelating capacity which corresponds to the increased levels of EDDHA in the nutrient medium. Adding radioiron to the exudates caused the appearance of a pale red color, indicating the presence of $Fe^{55, 59}$ EDDHA. The color reaction indicates that the chelate in the exudate was iron free previous to iron addition.

Data from experiments in which FeEDDHA was added to the nutrient medium (table II) show that plant exudates increase both in FeEDDHA and in total iron as the nutrient concentrations of FeEDDHA are increased. The iron concentrations (as Fe-EDDHA) in the exudates are very low when compared to total iron. This difference suggests the separation of significant quantities of iron from FeEDDHA at the root surface with iron-free EDDHA remaining in the nutrient.

Table III shows a decrease of FeEDDHA and total iron in the nutrient medium as affected by root absorption of decapitated sunflower plants. The loss of Fe (as FeEDDHA) was from 5.4 to 3.6 ppm, or 1.8 ppm. Iron chelating properties of the nutrient increased, however, showing at the end of the experiment that nutrient samples retained (against pH 8

TABLE II

CHELATED FE (FEEDDHA) AND TOTAL FE CONCENTRATIONS IN EXUDATE FROM DECAPITATED ZINNIA, SUNFLOWER, AND SOYBEAN PLANTS AS AFFECTED BY INCREASED LEVELS OF FEEDDHA IN NUTRIENT SOLUTION

NUTRIENT SOLUTION TREATMENTS	CHELATED FE AND TOTAL FE IN PLANT EXUDATES					
	ZINNIA		SUNFLOWER		SOYBEAN	
FEEDDHA PPM	FE (FEEDDHA) PPM*	FE (TOTAL) PPM	FE (FEEDDHA) PPM*	FE (TOTAL) PPM	FE (FEEDDHA) PPM*	FE (TOTAL) PPM
0	0.00	0.1	0.00	0.3	0.00	0.1
55	0.05	0.6	0.07	1.4	0.12	1.5
220	0.05	2.3	0.07	1.2	0.31	2.8
440	0.34	4.6	0.21	3.9	0.51	3.6
660	0.31	5.3	0.36	5.0	0.78	4.6

* Values have been adjusted to compensate for exudate optical density values: 0.0000 for zinnia; 0.0088 for sunflower; and 0.410 for soybean, obtained on the zero FeEDDHA nutrient treatment.

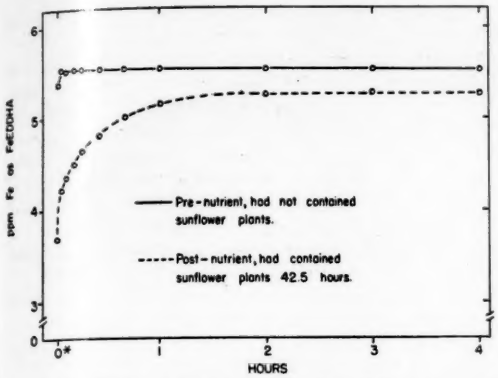


FIG. 4. Rate and extent of chelation of Fe, measured as FeEDDHA at 480 mμ, by a nutrient solution before and after containing decapitated sunflower plants.

and filtering) approximately 0.3 ppm $Fe^{55, 59}$. The exudate shows first a sharp rise, then a decline in total iron. Radioiron levels retained by exudate were low, indicating a low iron chelating capacity of the exudate. The progressive reduction of FeEDDHA in the nutrient medium (table III) might suggest the equivalent loss of both components of the chelate; but the concomitant increase in iron chelating capacity shows that iron-free EDDHA may have been left in the nutrient medium. Similar results were obtained in three confirmatory experiments.

To resolve this question, Fe was added to aliquots of the medium sampled at the beginning and at the end of 42.5 hours growth of sunflower plants. Figure 4 shows the restoration of the FeEDDHA complex after the addition of excess Fe to both solutions. This demonstrates the presence of considerable iron-free chelating agent in the medium.

DISCUSSION

The quantities of radioiron chelated by plant exudates were very low (table I) indicating the presence of only small amounts of chelating agent. However, there was even some radioiron held by the exudate from plants in zero EDDHA treatments. These values suggest the presence of natural chelators which are capable of holding iron soluble against high pH, heat, and filtering. The quantities of Fe held by natural chelators were deducted from the remaining exudate values for the respective plant exudates in order to determine radioiron chelated as FeEDDHA.

If calculations are made on a combining weight basis (55 g Fe to 360 g EDDHA = 1 : 6.5), the overall average for the 11 observations in table I indicates that 0.3 % of the EDDHA concentration in the nutrient medium can be found as iron chelate in the plant exudate. Broken down to individual species these observations range from 0.10 % to 0.45 %, with an average of 0.2 % for zinnia; 0.20 % to 0.33 %, with an average of 0.25 % for sunflower; and 0.27 % to 0.84 %, with an average of 0.53 % for soybean.

EDDHA entered roots from an iron deficient medium. It is therefore capable of entering roots in an iron-free form. This fact reinforces the possibility that this agent may enter roots as the chelate of various metals.

Small quantities of FeEDDHA were found in plant exudates. The iron chelate in the exudate suggests, but does not prove, that Fe penetrated the roots in this form. The quantities of chelated Fe are low when compared to total Fe. Calculations from table II show that on the average only 1 out of every 12 Fe atoms in the total plant exudate was in the form FeEDDHA. Expressed as a percentage of total Fe, the chelated Fe was 5.9 % for zinnia, 5.8 % for sunflower, and 12.5 % for soybean.

Large quantities of Fe were made available to the root by the metal chelate. In some cases the Fe concentration in the exudate was much higher than the corresponding concentration of Fe in the nutrient. Data from table III show that concentrations of Fe in the exudate range to nearly 20 times the Fe levels in the nutrient. On the average, the concentration of total Fe in the exudate is eight times greater than total Fe in the nutrient. The approximate total loss

TABLE III

CHANGES IN FE (FEEDDHA), TOTAL FE, AND CHELATING CAPACITY* OF NUTRIENT SOLUTION CONTAINING SUNFLOWER PLANTS AND CONCURRENT CHANGES IN TOTAL FE AND CHELATING CAPACITY OF PLANT EXUDATE

NUTRIENT AND EXUDATE SAMPLED AT: HRS	NUTRIENT SOLUTION			EXUDATE	
	FE (FEEDDHA) PPM	FE (TOTAL) PPM	FE CHELATING CAPACITY* PPM	FE (TOTAL) PPM	FE CHELATING CAPACITY* PPM
0	5.4	5.5	0.043	...	...
5	...	5.4	0.177	3	0.008
10.5	4.5	4.5	0.227	25	0.004
16.5	...	3.5	0.282	69	0.006
23.0	3.6	3.2	0.289	54	0.005
31.5	...	3.3	0.315	24	0.003
42.5	3.6	3.2	0.302	6	0.012

*In terms of $Fe^{55, 59}$ chelated

of Fe from the nutrient was 2 mg/l. One half of this amount was found in the 30 ml of exudate.

Comparison of Fe (FeEDDHA) in pre- and post-nutrient in figure 4 shows that pre-nutrient contained 5.4 ppm of chelated Fe before plants were placed in the nutrient. After 42.5 hours the sunflower plants had reduced this to 3.6 ppm of chelated Fe. This represents a loss of 1.8 mg of Fe from the liter of nutrient. If this quantity of iron had been accompanied by equivalent chelating agent, the nutrient should have lost 11 mg EDDHA. This was not the case. One hour after adding excess Fe^{+++} to the samples the complexing of iron free EDDHA remaining in the post-nutrient was nearly complete. Optical density readings at 3 and 4 hours were identical, showing equilibrium. The difference at equilibrium between chelated Fe values for the pre- and post-nutrient is 0.25 ppm Fe. On a combining weight basis (1:6.5), 1.6 ppm EDDHA would be required to chelate 0.25 ppm Fe. The quantity, 1.6 mg EDDHA, represents the loss of chelating agent from 1 liter of nutrient medium in 42.5 hours. This is a seventh the expected EDDHA loss if EDDHA had been absorbed with Fe in equivalent quantities.

The valence state and the form of Fe entering the root is unknown. If EDDHA entered in this case as FeEDDHA, then, on the average, the ratio of non-chelated Fe atoms entering sunflower roots to those entering as FeEDDHA would be approximately 6:1.

Experiments both with animals and plants are in general agreement with these results. Seeberg, et al (9) found that iron from iron chelates was absorbed from the gastrointestinal tract of anemic rats and utilized for hemoglobin regeneration at the same rate as iron from ferrous sulfate. Applied intravenously the iron was not readily available to the body. It would appear that iron is released from the chelate in rats by some mechanism in the gastrointestinal tract before being absorbed; in the blood stream the metal ions remain attached to the chelate. Krauss and Specht (4), studying the absorption of C^{14}-labeled FeEDTA by Scenedesmus cells, found that EDTA or some breakdown product was absorbed. However, their calculations showed insufficient EDTA present to account for the high iron levels. In a series of experiments, only enough EDTA to account for 1/50 to 1/15 of the iron was found in the Scenedesmus cells.

Wallace, et al (16) added $Fe^{59}N^{15}$ EDTA to nutrient cultures growing bush beans and found that the isotopes were fairly well distributed in most plant parts. Ratios of Fe^{59}/N^{15}EDTA indicated, however, that the uptake, translocation, and accumulation of iron and EDTA by plants may not have been equivalent. The ratio for the entire leaf was 1.41, for the stem 0.98, and for the entire plant 2.79. A very noticeable difference in the amounts of iron and chelating agent was observed in root analyses, where the ratio of Fe/EDTA was 6.13. This fact suggests that Fe was separated from the iron chelate at the root surface. A further study (16) with soybeans in sand culture containing FeEDDHA also revealed large amounts of Fe in the roots.

One of the most emphasized facts in literature on chelates has been the increase of iron in plant parts following iron chelate application. Although the generally accepted view has been that both components are absorbed together, it seems reasonable to assume that at least some of the many reported instances of high Fe levels in plants may be due to the separation of Fe from the chelate by the roots.

The uptake of non-equivalent quantities of chelate components by a few plants suggests the need for more extensive investigations among various plant families in order to determine how widespread this phenomenon may be. To expect all types of plants to respond alike in their interactions with factors relating to iron supply in a nutrient medium is not possible. Therefore, some plants may be expected to manifest only threshold responses to chelated Fe, whereas others may utilize chelated Fe very readily. And though conclusive demonstrations are not available at the present time, it may be that some plants will be found to absorb readily both components in equivalent quantities.

From these experiments it is concluded that zinnia, sunflower, and soybean plants do not absorb Fe and EDDHA in equivalent quantities, but that Fe is released to the roots, with most of the EDDHA remaining in the nutrient medium.

Summary

To study the absorption of metal chelate components, zinnia, sunflower, and soybean plants were grown in media containing ethylenediamine di(*o*-hydroxyphenylacetic acid), EDDHA, or the ferric chelate of this acid, FeEDDHA. Plants were decapitated and the exudates were collected for analyses of total iron, chelated iron, and chelating capacity.

Exudates from plants with roots in EDDHA contained small amounts of EDDHA. The average concentration was 0.3 % of that in the nutrient medium.

Exudate from plants with roots in FeEDDHA contained small amounts of FeEDDHA but relatively large amounts of total Fe. The average ratio of chelated Fe to total Fe was 1:12.

A time course experiment with sunflowers and FeEDDHA showed the progressive loss of Fe from the nutrient solution and the accumulation of Fe in the exudate. The FeEDDHA chelate delivered Fe to the root. As a result, the average total Fe in the exudate was eight times the average concentration of total Fe in the medium. With the loss of Fe from the nutrient, there was a seven fold increase in the iron chelating capacity of the nutrient. Analyses showed that the increase in chelating capacity was due to an increase in iron free EDDHA concomitant with Fe uptake by the roots. It is concluded, therefore, that these plant species selectively absorb Fe, the EDDHA remaining, for the most part, in the nutrient medium.

Acknowledgement

The authors wish to express their appreciation to Geigy Agricultural Chemicals, Division of Geigy Chemical Corporation, 89 Barclay Street, N. Y., for contributing the chelate and chelating agent used in this study.

Literature Cited

1. Heck, W. W. and L. F. Bailey. 1950. Chelation of trace metals in nutrient solution. Plant Physiol. 25: 573.
2. Holmes, R. S. and J. C. Brown. 1955. Chelates as correctives for chlorosis. Soil Sci. 80: 167–169.
3. Hutner, S. H., L. Provasoli, A. Schatz, and C. P. Haskins. 1950. Some approaches to the study of the role of metals in the metabolism of microorganisms. Proc. Amer. Phil. Soc. 94: 152–170.
4. Krauss, R. W. and A. W. Specht. 1958. Nutritional requirements and yields of algae in mass culture. In: Transactions of the Conference on the Use of Solar Energy—The Scientific Basis, Edwin F. Carpenter, ed. Vol. IV. Photochemical Processes. Pp. 12–26. University of Arizona Press, Tucson.
5. Kroll, H., M. Knell, J. Powers, and J. Simonian. 1957. A phenolic analog of ethylenediaminetetraacetic acid. Jour. Amer. Chem. Soc. 79: 2024.
6. Leonard, C. D. and I. Stewart. 1953. An available source of iron for plants. Proc. Amer. Soc. Hort. Sci. 62: 103–110.
7. Saywell, L. G. and B. B. Cunningham. 1937. Determination of iron, colorimetric *o*-phenanthroline method. Ind. Eng. Chem., Anal. Ed. 9: 67–69.
8. Schatz, A. and S. H. Hutner. 1949. An inert metal carrier for culture media. Abstracts of Papers, Soc. Amer. Bacteriologists. P. 34.
9. Seeberg, V. P., J. Hidalgo, and W. Wilken. 1954. Hemoglobin regeneration following oral administration of chelated iron. Science 119: 608–609.
10. Steinberg, R. A. 1953. Symptoms of molybdenum deficiency in tobacco. Plant Physiol. 28: 319–322.
11. Stewart, I. and C. D. Leonard. 1954. Chelated metals for growing plants. In: Mineral Nutrition of Fruit Crops, N. F. Childers, ed. Pp. 775–809. Horticulture Publications, New Brunswick, N. J.
12. Stewart, I. and C. D. Leonard. 1952. Chelates as sources of iron for plants growing in the field. Science 116: 564–566.
13. Stewart, I. and C. D. Leonard. 1952. Iron chlorosis—its possible causes and control. Citrus Magazine 14: 22–25.
14. Wallace, A. and C. P. North. 1953. Lime induced chlorosis. Calif. Agric. Exp. Sta. Circ. 7: 8–10.
15. Wallace, A., C. P., North, R. T. Mueller, L. M. Shannon, and N. Hemaidan. 1955. Behavior of chelating agents in plants. Proc. Amer. Soc. Hort. Sci. 65: 9–16.
16. Wallace, A., L. M. Shannon, O. R. Lunt, and R. L. Impey. 1957. Some aspects of the use of metal chelates as micronutrient fertilizer sources. Soil Sci. 84: 27–41.

CHLOROPHYLLASE ACTIVITY OF SPINACH CHLOROPLASTIN[1, 2]

CARMEN ARDAO[3] AND BIRGIT VENNESLAND

DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF CHICAGO, CHICAGO, ILL.

When spinach chloroplasts are treated with digitonin, a chlorophyll-lipoprotein complex is solubilized. Careful addition of ethanol to the clear green solution gives a green precipitate which is one of the components (factor 1) of cytochrome c photooxidase (1, 9, 10). Since factor 1 is a chlorophyll-lipoprotein complex, it may properly be called chloroplastin. The present paper is concerned with the determination of the chlorophyllase activity of this chloroplastin.

MATERIALS AND METHODS

Chlorophyll a was prepared according to Zscheile and Comar (14). It was dissolved in ethyl ether and stored at −15° C. All organic solvents were redistilled. The spectrophotometric observations were made with a Beckman recording spectrophotometer. Chloroplastin was the precipitate designated factor 1 prepared from spinach leaves as previously described (9). Two digitonin extractions were always made.

PREPARATION AND ASSAY OF CHLOROPHYLLASE FROM CHLOROPLASTIN. Chlorophyllase is the enzyme, first described by Willstätter and Stoll (13), which catalyzes the hydrolytic (or alcoholytic) cleavage of the phytol moiety from chlorophyll, giving free phytol and chlorophyllide. The assay procedure here described is a modification of the method of Weast and Mackinney (12), and is based on the change in solubility of pigment that occurs when the phytol moiety is removed from the chlorophyll molecule. Chlorophyll can be extracted from aqueous acetone by petroleum ether, but chlorophyllide remains in the acetone layer. The hydrolysis reaction is carried out in 66 % acetone in water. The chlorophyll naturally present in the leaf preparation may serve as enzyme substrate or the enzyme may be freed of chlorophyll by exhaustive extraction with aqueous acetone, after which chlorophyll is added back. In the present studies the latter alternative was preferred because of its higher accuracy.

For removal of chlorophyll, the moist precipitate of chloroplastin was suspended in about ten volumes of 80 % aqueous acetone and the suspension was filtered with suction. The acetone extraction was repeated 10 times or more if necessary. The residue was suspended in water (50 ml for material from 6 kg spinach), and the insoluble enzyme was recovered by centrifugation (5 min at 2500 × G). No trace of chlorophyllase activity was present in the aqueous extract. The enzyme was obtained in the form of a paste (about 1 g per kg spinach) containing about 80 per cent water. A bright red pigment which collected on the surface of the precipitate after centrifugation from water had no effect on enzyme activity. The amount of chlorophyllase obtained was the same whether the acetone extraction was carried out at room temperature or at 0° C. Almost complete loss of enzyme activity often occurred if the paste was thoroughly air-dried, and a moderate (20–50 %) loss occurred on freezing.

An aliquot of a solution of chlorophyll a in ethyl ether was measured into the bottom of a reaction flask, and the ether was removed by evacuation. (About 0.02–0.04 mg of chlorophyll was a convenient amount.) The chlorophyll residue was dissolved in 8 ml of 80 % acetone, and a weighed amount of enzyme paste was added with 2 ml of H_2O. The mixture was ground to a fine suspension with a glass rod. The stoppered flask was incubated in the dark with shaking for 3 hours at 22° C. After filtration of the suspension, a 5 ml aliquot of the filtrate was transferred to a glass-stoppered 10 ml graduated cylinder, and 5 ml of petroleum ether were added. The liquid layers were mixed by shaking and allowed to separate into a petroleum ether, acetone layer (usually 6.2 ml) on top and an aqueous acetone layer (usually 3.8 ml) below. Occasionally the acetone layers were turbid and had to be cleared by centrifugation. The visible spectrum of each layer was determined in a 1 cm cell. The amount of chlorophyll added to a particular reaction mixture was determined by making an identical reaction mixture without added enzyme, and carrying through the incubation and extraction exactly as in the case of the reaction with enzyme. Correction was thus automatically made for chlorophyll destroyed in the absence of enzyme during the incubation period. The record from a typical set of measurements is shown in figure 1. In the present studies, the complete spectrum from 400 to 700 mμ was recorded, to ensure the identity of the substances measured; but readings may be limited to the maxima in the red. To calculate the amount of chlorophyll a in the petroleum ether, the unit absorbancy used was 102 cm^2 mg^{-1} at the maximum of about 660 mμ. The unit

---

[1] Received August 19, 1959.

[2] Supported in part by a grant from the National Science Foundation to E. S. Guzman Barron of the Department of Medicine, University of Chicago.

[3] Present address: Instituto de Endocrinologia, Montevideo, Uruguay.

absorbancy represents the optical density or absorbancy of a solution containing 1 mg per ml of chlorophyll, observed with a light path of 1 cm. To calculate the chlorophyllide a in the acetone, the unit absorbancy used was 89 cm^2 mg^{-1} at the maximum of about 663 mμ. Chlorophyllide was calculated as mg chlorophyll. The assumption was made that the extinction coefficient of chlorophyllide in aqueous acetone was the same as that of chlorophyll in the same solvent. The unit absorbancy values of 102 and 89 for chlorophyll a in petroleum ether and in aqueous acetone were determined experimentally. The reference value employed was the unit absorbancy of chlorophyll a in pure ether = 102 at 660 mμ, as reported by Zscheile and Comar (14). Mackinney (7) reported a value of 90 for anhydrous ether, 93.4 for U.S.P. ether, and 82 for aqueous acetone. Harris and Zscheile (3) reported that the red absorption maximum of chlorophyll a in pure acetone is 83 % of the red absorption maximum in ether.

Not shown in figure 1 are the absorbancies of the solvent layers from a blank determination in which the incubation was carried out with enzyme alone without added chlorophyll. If the chlorophyll had been completely removed from the enzyme, there was no contribution to light absorbancy at 660 mμ. It was important to check this point with every enzyme preparation, because prolonged incubation with aqueous acetone may remove pigment resistant to removal in a brief extraction period. Other substances which absorb light of longer wave lengths may also be extracted into the acetone, particularily if the method is applied to leaf preparations different from chloroplastin. Though these other substances have no well-defined absorption peaks, they may take a considerable contribution to the total absorbancy at 660 mμ. If precautions are taken to carry out appropriate blank determinations, however, the method is applicable to a wide range of preparations.

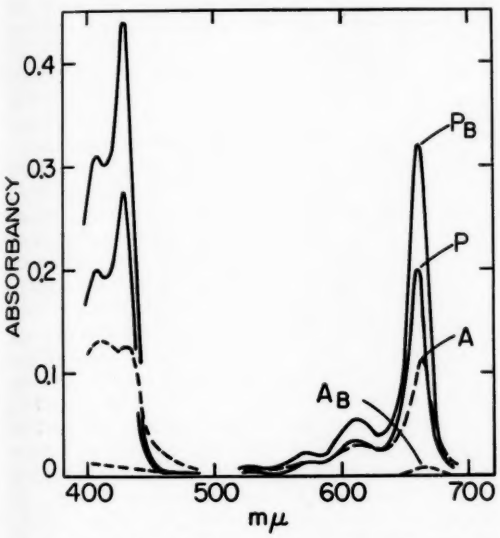


FIG. 1. Record of light absorbancy of petroleum ether and acetone layers from chlorophyllase assay procedure. Absorbancy = log I_0/I, where I_0 = intensity of incident light, and I = intensity of transmitted light.

Incubation was with 100 mg wet paste from chloroplastin for 3 hours at 22° C.

P = absorbancy of petroleum ether layer after incubation with enzyme. P_B = absorbancy of petroleum ether layer after incubation without enzyme. A = absorbancy of acetone layer after incubation with enzyme. A_B = absorbancy of acetone layer after incubation without enzyme.

Blank determinations carried out with the enzyme preparation alone without added chlorophyll did not give any light absorption between 600 and 700 mμ.

Results and Discussion

The numerical values calculated from the spectrophotometric data of figure 1 are given in the first line of table I. This table also includes the results of two sets of measurements made with varying chlorophyll concentrations, and another set of duplicate determinations carried out at two different temperatures. The data given in the table were selected from more than 100 separate experiments to illustrate typical assay results and the degree of duplicability. Recovery of total pigments ranged from 70 to 97 %, with an average of about 80 %. This was considerably better than the recovery we could achieve with the recently described chromatographic procedures for assaying chlorophyllase (2, 11).

Because of the variable and incomplete recovery of pigment, it is not a straightforward matter to express the enzyme activity numerically. In table I, the per cent hydrolysis has been calculated both on the basis (A) of the decrease of pigment in the petroleum ether layer, and (B) of the increase of pigment in the acetone layer. The difference between these two numbers is of course larger when the per cent recovery is smaller. The per cent hydrolysis calculated in either fashion was linearly proportional to the amount of enzyme assayed within the range from 100 to 200 mg of enzyme paste from chloroplastin. This proportionality is a convenient rule of thumb, but only reflects the fact that the deviations from linearity in this activity range are not greater than the fluctuations of duplicate determinations. The data in table I show that the per cent hydrolysis observed in a given time period is almost independent of initial chlorophyll concentration in the range tested. This suggests first order kinetics. When the reaction was followed with time, however, the results confirmed the conclusion of Weast and Mackinney (12) that the enzyme reaction rate decreased with time, but the kinetics were neither first nor second order. The kinetics are obviously complex, and may reflect a basically first order reaction which is inhibited by a

TABLE I

RECOVERY OF PIGMENT IN ASSAY

EXPERIMENT	CHLOROPHYLL		CHLOROPHYLLIDE*	RECOVERY**	% HYDROLYSIS***	
	ADDED MG	RECOVERED MG	RECOVERED MG	%	A	B
Data from fig 1	0.038	0.024	0.0094	88	37	25
Effect of chlorophyll Concentration 1)	0.033	0.017	0.013	91	48	39
	0.298	0.182	0.064	83	39	21
2)	0.032	0.011	0.014	78	67	44
	0.065	0.028	0.020	74	57	31
	0.103	0.053	0.027	78	49	26
Effect of temperature 20°	0.033	0.015	0.011	79	55	33
	0.032	0.013	0.013	81	59	41
40°	0.032	0.007	0.024	97	78	75
	0.033	0.008	0.022	91	76	67

* Calculated as chlorophyll

** $100 \times \frac{\text{chlorophyll recovered} + \text{chlorophyllide recovered}}{\text{chlorophyll added}}$

*** % Hydrolysis. A. Calculated on basis of decrease in chlorophyll. B. Calculated on basis of formation of chlorophyllide. All reactions carried out with 100 mg wet paste from chloroplastin.

reaction product and/or accompanied by progressive enzyme inactivation.

The claim of Mayer (8) that chlorophyllase is inactivated by washing with water and reactivated by adding salts could not be confirmed. The only effect of extraction with water, as applied in preparing the enzyme for assay, was the removal of soluble materials with no enzyme activity. Adding phosphate buffer of pH close to neutrality did not increase enzyme activity but gave higher blank values, so buffer was generally omitted from the assay system.

No alcoholysis of chlorophyll could be obtained with our chloroplastin preparations. This is in agreement with the observations of Weast and Mackinney who found only a trace of alcoholysis reaction in spinach preparations (12).

The present studies were prompted by the possibility that the activity of factor 1 in the cytochrome photooxidase system might be dependent on the formation of a relatively unstable chlorophyllide from the chlorophyll present in the preparation. Krossing (6) had concluded previously that the chlorophyllase activity of leaves was localized in the chloroplasts, so it seemed quite likely that the chlorophyllase might be present in factor 1 itself. The present studies confirmed this supposition. Careful measurements showed, however, that factor 1 contained no detectable chlorophyllide, nor was there any formation of chlorophyllide during incubation of factor 1 for 24 hours at room temperature, either in digitonin solution or in aqueous suspension under conditions used for the assay of cytochrome photooxidase. The presence of digitonin was shown to have no effect on the chlorophyllase assay. Thus, though the chlorophyllase is solubilized with the chlorophyll by the digitonin, chlorophyll and enzyme remain firmly attached under circumstances which permit no trace of enzyme action. Only after the chlorophyll has been removed from the lipoprotein by the action of suitable organic solvents is the enzyme capable of causing chlorophyll hydrolysis. The dependence of chlorophyllase action on the presence of organic solvents resembles the similar dependence of phosphatidase c. The organic solvent may act to promote appropriate contact between enzyme and substrate (4, 5). In the intact chloroplastin preparation itself one must conclude either that the enzyme is inactive or that the chlorophyll is bound in such a way as to be protected from enzyme action. It is impossible at present to distinguish between these two possibilities, because the solvents known to permit enzyme action also cause at least partial extraction of the endogenous chlorophyll. Aqueous suspensions of externally added chlorophyll are not attacked by chloroplastin, but this can be attributed to absence of contact between enzyme and substrate.

A final question to which an answer was sought was whether all the chlorophyllase of the leaf could be accounted for in the chloroplastin fraction. Because of the difficulty of assaying chlorophyllase quantitatively, particularly in crude leaf homogenates, a conclusive answer to this question could not be obtained. The most reliable procedure for determining the relative activity of two different enzyme preparations appeared to involve the determination, by trial and error, of those amounts of enzyme giving

TABLE II

RELATIVE CHLOROPHYLLASE ACTIVITY

PREPARATION	% HYDROLYSIS IN 3 HRS**
Acetone powder from whole leaf, 1 g dry wt	33
Acetone powder from washed chloroplasts, 100 mg dry wt	44
Residue* from washed chloroplasts, 100 mg wet wt	30
Residue* from factor 1, 100 mg wet wt	41

* Extracted with aqueous acetone until free of chlorophyll, then extracted with water. Water content, 80 %.
** Calculated on the basis of disappearance of pigment from the petroleum ether layer.

about the same per cent hydrolysis of the same amount of chlorophyll. Table II contains the results of some assays of different preparations from the same batch of leaves. These enzyme measurements were performed with the object of getting accurate values for comparative purposes. Assays of the dried acetone powders of whole leaves and of washed chloroplasts should be comparable, and showed that the powder from chloroplasts was about thirteen times as active as the powder from whole leaves. The ratio of the chlorophyll content of the whole leaves to that of the chloroplasts, calculated on a dry weight basis, was about 1 to 15. The wet paste from chloroplastin was somewhat higher in activity on a dry weight basis than the wet paste from chloroplasts. These data are compatible with the possibility that in the mature spinach leaf, chlorophyllase is localized entirely in the chlorophyll-lipoprotein complex of the chloroplasts, since most of the chlorophyll of the chloroplasts can be solubilized in the form of chloroplastin, and since the chloroplastin contains most though not all the dry weight of the chloroplasts. Because of the inaccuracy of the assays, however, more work would be required to establish this site as the exclusive location of the enzyme.

SUMMARY

A method for the assay of chlorophyllase has been described. The enzyme has been shown to be present in the chlorophyll-containing component of cytochrome c photooxidase of spinach leaves in sufficient quantity to suggest that all the chlorophyllase of the mature leaf may be present in a chlorophyll-lipoprotein complex. The chlorophyllase of digitonin-solubilized chloroplastin has no hydrolytic effect on the chlorophyll bound to lipoprotein in the preparation, but acts only after the chlorophyll has been separated by suitable organic solvents.

LITERATURE CITED

1. BISHOP, N. I., H. NAKAMURA, J. BLATT, and B. VENNESLAND. 1959. Kinetics and properties of cytochrome c photooxidase of spinach. Plant Physiol. 34: 551–557.
2. GAGE, R. S. and S. ARONOFF. 1956. Chlorophyllase in soybean. Plant Physiol. 31: 477–478.
3. HARRIS, D. G. and F. P. ZSCHEILE. 1943. Effects of solvent upon absorption spectra of chlorophylls a and b; their ultraviolet absorption spectra in ether solution. Bot. Gaz. 104: 515–527.
4. KATES, M. 1957. Effects of solvents and surface-active agents on plastid phosphatidase c activity. Canadian Jour. Biochem. Physiol. 35: 127–142.
5. KATES, M. and P. R. GORHAM. 1957. Coalescence as a factor in solvent stimulation of plastid phosphatidase c activity. Canadian Jour. Biochem. Physiol. 35: 119–126.
6. KROSSING, G. 1940. Versuche zur Localisation einiger Fermente in den verschiedenen Zellbestandteilen der Spinatblätter. Biochem. Zeits. 305: 359–373.
7. MACKINNEY, G. 1941. Absorption of light by chlorophyll solutions. Jour. Biol. Chem. 140: 315–322.
8. MAYER, H. 1930. Untersuchungen über die Chlorophyllase. Planta 11: 294–330.
9. NIEMAN, R. H., H. NAKAMURA, and B. VENNESLAND. 1959. Fractionation and purification of cytochrome c photooxidase of spinach. Plant Physiol. 34: 262–267.
10. NIEMAN, R. H. and B. VENNESLAND. 1959. Photoreduction and photooxidation of cytochrome c by spinach chloroplast preparations. Plant Physiol. 34: 255–262.
11. SIRONVAL, C. 1954. Mesure de l'activité de la chlorophyllase par chromatographie sur papier. Physiol. Plantarum. 7: 523–530.
12. WEAST, C. A. and G. MACKINNEY. 1940. Chlorophyllase. Jour. Biol. Chem. 133: 551–558.
13. WILLSTÄTTER, R. and A. STOLL. 1913. Untersuchungen über Chlorophyll. Berlin.
14. ZSCHEILE, F. P. and C. L. COMAR. 1941. Influence of preparative procedure on the purity of chlorophyll components as shown by absorption spectra. Bot. Gaz. 102: 463–481.

IMPAIRMENT OF RESPIRATION, ION ACCUMULATION, AND ION RETENTION IN ROOT TISSUE TREATED WITH RIBONUCLEASE AND ETHYLENEDIAMINE TETRAACETIC ACID[1, 2]

J. B. HANSON

DEPARTMENT OF AGRONOMY, UNIVERSITY OF ILLINOIS, URBANA

Ribonuclease has been reported to impair protein synthesis (1, 2), growth (2), and ion accumulation (15, 24) in plant tissue. Oxidative phosphorylation by plant mitochondria is uncoupled by RNase[3] (8); however, this enzyme has not been found to affect root respiration (1, 8).

The experiments reported here represent a further study of the effects of RNase on respiration and ion accumulation by root tissue. Respiration and ion retention proved to be impaired by exogenous RNase. In addition, EDTA was found to produce similar responses, presumably by removal of divalent ions which normally protect polyribonucleotides from endogenous RNase.

METHODS AND MATERIALS

PLANT MATERIALS: Soybean seed (*Glycine max;* var. Hawkeye) and hybrid corn seed (*Zea mays* L., var. WF9 x M14) were lightly dusted with a fungicide (Spergon; U.S. Rubber Co.) and germinated for 3 days at 28° C in darkness in vermiculite moistened with tap water. Initially, the tip 1.5 cm of the primary roots was used; later the roots were sectioned into three successive 0.5 cm sections. The tissue was rinsed in deionized water, blotted, and rapidly weighed to the nearest milligram before use.

RESPIRATION AND ION ACCUMULATION: Oxygen consumption was measured at 29° C by standard manometric techniques (27). Air was gas phase. The tissue was placed in 2.0 ml of 10^{-3} M potassium phosphate, pH 6.0. Treatments were in duplicate or triplicate. Vessel additives are given in the tables. The EDTA was added as the potassium salt, pH 6.0. Protease-free, five times recrystallised RNase was obtained from the Sigma Chemical Co.

Phosphate accumulation from the Warburg vessel media was measured during the respiratory determinations. Potassium accumulation was largely determined in parallel experiments using 50 ml Erlenmeyer flasks on a shaker at room temperature. In each case the tissue was placed in 10^{-3} M potassium phosphate buffer (pH 6.0) labeled with P^{32} or Rb^{86}. The Rb ion serves as a tracer for K accumulation in short-term experiments with root tissue (14). At the termination of the accumulation period, the exchangeable Rb+K ion was removed from the tissue in two out of four flasks in each treatment by rinsing the tissue with deionized water and placing it in ice-cold 10^{-3} M $CaCl_2$ for 40 minutes. Tissues were rinsed, digested and assayed as previously described (14). Exchangeable Rb+K was calculated from the difference in radioactivity between tissue subjected to exchange with Ca, and tissue which was only rinsed with deionized water.

LEAKAGE OF IONS: Root tip tissue was placed in 125 ml Erlenmeyer flasks containing 10 ml of 1% sucrose with 10^{-3} M potassium phosphate buffer labeled with Rb^{86}. After 2 hours on a shaker at room temperature, the tissue was rinsed and exchangeable ions removed in unlabeled 10^{-3} M phosphate buffer containing 10^{-2} M $CaCl_2$ as above. Next, the tissue was incubated for 1 hour on the shaker in the same sucrose-buffer medium as during the uptake phase but without the Rb^{86} label. Ribonuclease and EDTA were added as indicated in the tables. The tissue was rinsed with water, ashed, and residual radioactivity determined as above.

OXIDATIVE PHOSPHORYLATION OF MITOCHONDRIA: Mitochondria were isolated from 1.5 cm sections of soybean root tips and the oxidative phosphorylation determined as previously described (8, 18). Alpha-ketoglutarate was used as substrate.

ANALYSIS FOR NUCLEOTIDES AND NUCLEIC ACIDS: The analytical procedures were adapted from those previously described for analysis of mitochondria (8). Concentrations were determined from 260 to 290 mμ absorption in the Beckman DU spectrophotometer, using AMP and yeast RNA as standards. Nucleotides diffusing from the tissue were estimated as AMP

---

[1] Received August 19, 1959.

[2] This research was supported by grants from the National Science Foundation and the Atomic Energy Commission.

[3] Abbreviations: EDTA, ethylenediamine tetraacetic acid; RNA, ribonucleic acid; RNase, ribonuclease. AMP, CMP, GMP, UMP, monophosphate nucleotides of adenosine, cytidine, guanosine, and uridine.

on the vessel contents at the end of the experiment or preincubation. The weighed root tips from the experiment were homogenized for 2 minutes in 3 ml of water with an ice-jacketed glass homogenizer, and transferred with 3 ml of water to centrifuge tubes. Perchloric acid was added to 0.2 *N* final concentration, and the precipitate sedimented by centrifugation. The pellet was twice resuspended in 0.2 *N* $HClO_4$ and recentrifuged. Cold-acid soluble nucleotides were estimated on the combined supernatants. The absorption spectrum of the cold $HClO_4$ extract from soybean roots showed a smooth increase in optical density from 290 mμ to a pronounced shoulder at 260 mμ; a small increase occurred between 260 to 250 mμ, followed by a decline to 235 mμ. Total nucleic acids were extracted from the washed and defatted precipitate with hot perchloric acid as described for mitochondria (8).

Assays for Enzymatic Degradation of Endogenous and Yeast RNA: About 240 root sections were homogenized for 2 minutes with 15 ml of 0.5 M sucrose in an ice-jacketed glass homogenizer with a Teflon pestle. The homogenate was cleared of cell debris and nuclei by centrifuging at 800 × G for 5 minutes. The supernatant solution was filtered through a small pad of glass wool to catch any fluffy pieces of cell wall debris. The cleared homogenate was adjusted to pH 6.5 with NaOH and used directly as a source of enzyme. The protein concentration was determined by the method of Lowry, et al (17), on aliquots precipitated and washed in 5 % trichloroacetic acid.

Degradation of endogenous and yeast RNA was determined by incubating 1 ml of cleared homogenate with the additives indicated in table XII. Final volume was 2 ml; incubation was for 30 minutes at 30° C; pH was 6.5; determinations were in duplicate. One set of tubes was held in ice to establish the initial level of nucleic acid.

At the termination of incubation, the tubes were placed in ice. Perchloric acid was added to 0.2 *N*. The precipitate was sedimented, washed, and assayed for nucleic acid as above. Experimentation has shown no detectable deoxyribose in comparable cleared homogenates; the endogenous nucleic acid is reported as RNA.

Table I

Inhibitory Effect of Ribonuclease on Root Tip Respiration

Root tips	Buffer	Qo_2(FW) Control	RNase	% Inhibition
Corn	None	552	525	5
	K Phosphate	702	572	19
Soybean	None	639	555	13
	K Phosphate	696	598	14

Twenty root tips were placed in Warburg vessels in 2 ml of deionized water or 10^{-3} M potassium phosphate (pH 6.0) with and without 1 mg RNase/ml. Respiration rates were measured between 70 and 100 minutes after placing roots in the vessels. Qo_2(FW) = μl O_2/hr/gm fresh weight.

Table II

Respiration Rate of Soybean Root Tips as Function of Treatment with Ribonuclease or EDTA

Vessel additive	Qo_2(FW) 0-25 Min	130-190 Min
Buffer	605	735
+ RNase	678	640
+ EDTA	913	493
+ RNase and EDTA	897	372

Twenty root tips were placed in Warburg vessels with 10^{-3} M potassium phosphate buffer (pH 6.0) with indicated additives. Final concentrations: RNase, 1 mg/ml; EDTA, 5 × 10^{-3} M.

Results

In the previous report on the activity of mitochondria treated with RNase (8), it was noted that corn roots treated with RNase showed no significant impairment of respiration in 2 hours. Table I shows that respiration declines after about one hour if the treatment is made in the presence of potassium phosphate buffer. Soybean root tips were sensitive to RNase in either water or buffer. The increased respiration of tissue in buffer, which is probably a manifestation of the widely described salt respiration, was also obtained with KCl. In subsequent experiments soybean root tips were generally more sensitive to RNase or EDTA treatment than were corn root tips. Experiments were largely done with soybeans. Occasional experiments were done with corn to make certain the results were valid for more than one species.

Table II compares the effects of RNase and EDTA on soybean root tip respiration. The application of EDTA was suggested by the reports of Ts'o (26) and West (30) showing that the RNA of the microsomal fraction of plant tissue is subject to degration by endogenous enzymes if treated with potassium ion or EDTA. Removal of the principal divalent ions of the cell, calcium and magnesium, by substitution or chelation, causes a dissociation of the ribonucleoprotein particles and appears to expose the RNA to degradation. The degradation can be largely halted by Ca or Mg.

In the initial minutes after application, both RNase and EDTA elicited increases in root respiration reminiscent of the increase following RNase application to mitochondria (8). EDTA has previously been reported to increase soybean leaf respiration (29). After 2 hours, respiration declined below the control levels. The EDTA was much more effective than RNase in both the initial respiratory burst and

TABLE III

EFFECT OF KCl ON RESPIRATION AND NUCLEOTIDE CONTENT OF SOYBEAN ROOTS

SOLUTION	Qo_2 (FW)	NUCLEOTIDES			
		DIFFUSIBLE	SOLUBLE	POLY-	TOTAL
		μg/gm fr. wt.			
Buffer (pH 6.0)	603	84	626	3,613	4,323
+ 10^{-2} M KCl	670	107	606	3,654	4,358
+ 10^{-1} M KCl	433	224	520	3,343	4,087

Twenty root tips were placed in 125 ml Erlenmeyer flasks with 10 ml of 10^{-3} phosphate buffer containing KCl as indicated. After 2 hours on a shaker at room temperature duplicate lots were analyzed for nucleotides. In a separate experiment the respiration rates after pretreatment were determined in 10^{-3} M phosphate buffer.

subsequent inhibition; the combination of EDTA and RNase produced the greatest impairment of respiration.

High concentrations of KCl impaired respiration after periods of 2 to 3 hours. Table III shows this effect and provides a balance sheet on the nucleotides. Three arbitrary classes of nucleotides are described: diffusible nucleotides are those which leak from the cells into the medium; soluble nucleotides are those which can be extracted from root homogenates in cold 0.2 *N* $HClO_4$; polynucleotides are those which can be extracted from the residue in hot 0.5 *N* $HClO_4$. The latter classification includes DNA (20). The 0.1 M KCl induced a degradation of polynucleotides and caused a loss of soluble nucleotides. Part of the lost nucleotides diffused from the tissue, and part disappeared (presumably through degradation of purine and pyrimidine bases, since nucleotides were measured by ultraviolet absorption).

Experiments (table IV) with corn root tips show that EDTA and RNase induced the same type of change in nucleotide content as did high concentrations of KCl. KCl was added to control treatments to prevent confounding the EDTA effect with the effect of K ion used in neutralization.

Table V shows that low concentrations of Ca ion would partially reverse the deleterious effects of EDTA on respiration and nucleotide content. Magnesium would also protect respiration.

The addition of monophosphate nucleotides with Ca and Mg gave essentially the same respiration response as Ca or Mg alone; both the KCl and EDTA treated tissue showed increased respiration (table VI).

TABLE V

EFFECT OF CALCIUM ION ON RESPIRATION AND POLYNUCLEOTIDE CONTENT OF SOYBEAN ROOT TIPS

VESSEL ADDITIVES	Qo_2 (FW)	NUCLEOTIDES	
		DIFFUSIBLE	POLY-
	90-150 min	*μg/gm fr. wt*	
KCl, 5×10^{-3} M	672	115	2,770
+ $CaCl_2$, 10^{-3} M	728	107	2,952
EDTA, 2×10^{-3} M	250	896	1,905
+ $CaCl_2$, 10^{-3} M	377	615	2,338

Experimental conditions as in table IV.

The decreased tissue respiration attendant on EDTA application was also reflected in the oxidative and phosphorylative capacity of mitochondria isolated from the tissue (table VII). The P/O ratios of mitochondria from the control (KCl-treated) tissue were surprisingly low; they varied from 0.5 to 1.3 in five experiments. Possibly some degradative metabolism in excised tissue during pretreatment was detrimental to the mitochondria.

TABLE IV

INHIBITION OF RESPIRATION AND LOSS OF NUCLEOTIDES FROM CORN ROOT TIPS TREATED WITH EDTA AND KCl

VESSEL ADDITIVES	Qo_2 (FW)	NUCLEOTIDES			
		DIFFUSIBLE	SOLUBLE	POLY-	TOTAL
	90-120 min		*μg/gm fr. wt*		
KCl, 0.0075 M	573	41	1,415	2,230	3,686
EDTA, 0.003 M	520	265	1,250	2,035	3,550
KCl, 0.025 M	566	94	1,173	1,970	3,237
EDTA, 0.01 M	260	553	658	1,720	2,931
KCl, 0.0075 M	645	43	1,440	2,043	3,526
+ RNase, 1 mg/ml	513	258	1,250	2,055	3,563
EDTA, 0.003 M	600	252	1,340	1,980	3,572
+ RNase, 1 mg/ml	529	258	1,275	1,673	3,206

Respiration measured in duplicate on 20 root tips in 10^{-3} M phosphate buffer (pH 6.0) with indicated additives. Nucleotide analyses made on the vessel contents and tissue after 2 hours.

TABLE VI

EFFECT OF DIVALENT IONS AND NUCLEOTIDES ON RESPIRATION OF EDTA-TREATED ROOT TIPS

ROOT TIPS	PRETREATMENT	VESSEL ADDITIVES	Qo_2(FW)
			90-120 min
Soybean	KCl, 0.005 M (60 min)	Buffer	464
		+ Ca, Mg, nucleotides	555
	K-EDTA, 0.002 M	Buffer	263
		+ Ca, Mg, nucleotides	408
Corn	KCl, 0.025 M (90 min.)	Buffer	564
		+ Ca, Mg, nucleotides	675
	K-EDTA, 0.01 M	Buffer	284
		+ Ca, Mg, nucleotides	366

Respiration determined in 10^{-3} M phosphate buffer, pH 6.0 with indicated additives. For pretreatment roots were placed in indicated solutions with 10^{-3} M phosphate buffer, pH 6.0, on a shaker at room temperature. Calcium and Mg were added as chlorides, 10^{-3} M. One mg each of AMP, CMP, GMP, and UMP was added to each vessel.

TABLE VII

INHIBITED OXIDATION AND PHOSPHORYLATION OF MITOCHONDRIA ISOLATED FROM ROOT TIPS TREATED WITH EDTA

PRETREATMENT SOLUTION	TISSUE Qo_2(FW)	MITOCHONDRIA Qo_2(N)	MITOCHONDRIA P/O
KCl, 2×10^{-2} M	507	431	0.77
EDTA, 10^{-2} M	285	133	0.00

About eight grams of soybean root tips were placed in 50 ml of 10^{-3} M phosphate buffer with indicated additives and agitated on a shaker at room temperature for 1.5 hours. Solutions were renewed after 45 minutes. Tissue respiration determined on 20 root tips. Mitochondria were isolated from the remaining tissue.

TABLE VIII

LOSS OF ACCUMULATED RUBIDIUM FROM SOYBEAN ROOTS DUE TO RIBONUCLEASE TREATMENT

TREATMENT	Rb^{86}	DIFFUSIBLE NUCLEOTIDE
	c/m/20 roots	*μg/20 roots*
Initial	962	...
Control	895	16
RNase	601	106

Root tips were incubated for 2 hours in 0.001 M potassium phosphate (pH 6.0) labeled with Rb^{86}. Exchangeable ions were removed with Ca and the root tips incubated in phosphate buffer with and without 1 mg/ml RNase for 2 hours.

TABLE IX

LOSS OF NUCLEOTIDES FROM SOYBEAN ROOT SECTIONS DUE TO EDTA TREATMENT

SOLUTION	ROOT SECTION CM FROM TIP	NUCLEOTIDES DIFFUSIBLE	NUCLEOTIDES SOLUBLE	NUCLEOTIDES POLY-	NUCLEOTIDES TOTAL
		μG/GM FR. WT			
Buffer	0.0-0.5	36	796	9,960	10,792
	0.5-1.0	57	319	1,675	2,051
	1.0-1.5	70	189	828	1,087
+ 3×10^{-3} M EDTA	0.0-0.5	206	640	9,570	10,416
	0.5-1.0	311	160	1,320	1,791
	1.0-1.5	225	117	645	987
		EDTA treatment as % of control			
	0.0-0.5	572	80	96	97
	0.5-1.0	546	50	79	87
	1.0-1.5	322	62	78	91

Duplicate samples of 30 root sections were incubated in 10^{-3} M phosphate buffer (pH 6.0) with and without EDTA for 1.5 hours at room temperature on a shaker, followed by analysis for nucleotides as described in the text.

TABLE X

EFFECT OF EDTA ON Rb^{86} RETENTION BY SECTIONS OF SOYBEAN ROOT

SOLUTION	ROOT SECTION	RADIOACTIVITY	EDTA AS % OF CONTROL
	cm from tip	*c/m/gm fr. wt*	
Buffer 10^{-3} M	0.0-0.5	12,600	
	0.5-1.0	6,000	
	1.0-1.5	3,200	
+ EDTA, 2×10^{-3} M	0.0-0.5	2,200	17
	0.5-1.0	1,900	32
	1.0-1.5	1,300	41

Root sections incubated on a shaker for 2 hours in potassium phosphate buffer, pH 6.0, labeled with Rb^{86}. This tissue was rinsed and exchangeable ion removed with 10^{-2} M $CaCl_2$ in unlabeled buffer. The tissue was next placed in the indicated solutions for 1 hour on a shaker at room temperature, rinsed, ashed, and counted.

The increase in diffusible nucleotides subsequent to treating the roots with RNase, EDTA or high concentrations of KCl suggested that the capacity of cell membranes to retain solutes had been impaired. This was directly confirmed by studying the loss of previously accumulated Rb^{86} (table VIII). RNase altered the cell membranes such that the ion leaked from the tissue. As will be shown later, EDTA produced the same effect.

Studies were made with successive 0.5 cm sections of soybean roots to relate the effect of EDTA to mean cell ontogeny. The 0.5 to 1.0 cm section encompasses the main region of cell expansion, and corresponds to the region where ion accumulation per cell, or per unit protein (4, 9) and mitochondrial activity (18) are greatest. The expanding zone of the root is also a region where the mitochondria and other membranous materials are rapidly increasing, and the amount of microsomal RNA is rapidly decreasing (18).

Table IX gives the nucleotide content as a function of EDTA treatment. The greatest leakage occurred in the tip section; the greatest loss of soluble nucleotide in the second (0.5-1.0 cm) section; the greatest degradation of polynucleotide in the 0.5 to 1.5 cm region. Since there appears to be some catabolism of nucleotides capable of diffusing from the tissue, (thus masking the relative loss of membrane semi-permeability) observations were made of the loss of accumulated Rb^{86} as a function of tissue maturity (table X). The meristematic tip region was the most sensitive.

Respiration and K accumulation were most sensitive to EDTA in the 0.5 to 1.0 cm sections (table XI). Phosphate accumulation was most affected in the tip. There was a pronounced increase in exchangeable cation with EDTA pretreatment; the greatest increase was in the expanding zone. The increase can be largely ascribed to loss of exchangeable divalent ions by chelation, but may in part result from increased negative valence at the protoplast surface due to cleavage of phosphate linkages in nucleic acid. A similar increase in exchangeable cation can be obtained with RNase application (data to be published separately).

The capacity of homogenates to digest endogenous and yeast RNA is given in table XII. The addition of EDTA accelerates the degradation of endogenous RNA over the KCl control. The greatest acceleration over control occurred in the tip, but as shown by the homogenate analyses, the higher substrate levels of the tip section may have been responsible for this result. If yeast RNA was added, the highest specific rate was in the 0.5 to 1.0 cm section, followed closely by the 1.0 to 1.5 cm section.

The declining RNA/protein ratio with distance from the tip suggests that the endogenous RNase is partially active during cell expansion and maturation.

TABLE XI

EFFECT OF EDTA PRETREATMENT ON RESPIRATION AND PHOSPHATE AND POTASSIUM ABSORPTION BY SECTIONS OF SOYBEAN ROOT TIPS

PRETREATMENT ADDITIVE	ROOT SECTION	Qo_2 (FW)	P ABSORPTION	Rb+K ABSORBED	
				EXCHANGEABLE	ACCUMULATED
	cm from tip	*0-120 min*	*μ moles/gm/hr*	*μ moles/gm*	*μ moles/gm/hr*
KCl, 5×10^{-3} M	0.0-0.5	1,110	0.38	1.66	4.40
	0.5-1.0	399	0.41	0.64	2.16
	1.0-1.5	348	0.34	0.89	1.34
EDTA, 2×10^{-3} M	0.0-0.5	733	0.11	4.67	2.27
	0.5-1.0	101	0.18	2.06	0.19
	1.0-1.5	181	0.19	2.04	0.36
			EDTA treatment as % of control		
	0.0-0.5	66	29	281	52
	0.5-1.0	25	44	322	9
	1.0-1.5	52	56	229	27

Pretreatment for 90 minutes on shaker at room temperature with KCl or EDTA in 10^{-3} M potassium phosphate. Respiration and phosphate absorption determined for 2 hours in Warburg vessels. Uptake of K labeled with Rb^{86} was for 2 hours on shaker at room temperature.

TABLE XII

DEGRADATION OF ENDOGENOUS AND YEAST RNA DURING INCUBATION OF HOMOGENATES FROM SUCCESSIVE SECTIONS OF SOYBEAN ROOT TIPS

ROOT SECTION	HOMOGENATE		RNA	RNA DEGRADATION BY HOMOGENATE		
	PROTEIN	RNA	PROTEIN	+ KCl	+ EDTA	+ RNA
cm from tip	*mg/gm fr. wt*	*mg/gm fr. wt*		*μg RNA/mg protein/hr*		
0.0-0.5	37.20	8.31	0.22	99	229	936
0.5-1.0	7.36	1.35	0.18	100	177	1,861
1.0-1.5	5.76	0.89	0.15	66	187	1,649

Homogenates cleared of cell debris and nuclei as described in methods, and incubated with 5×10^{-3} M KCl, 2×10^{-3} M EDTA or 1 mg/ml yeast RNA. Activity is expressed on the basis of homogenate protein.

but a concurrent synthesis of protein known to occur with root cell expansion (3, 28) would produce the same result.

The dependence of the EDTA effect on temperature is shown in table XIII. The sensitive tissue between 0.5 to 1.5 cm was used in these experiments. Pretreatment of the tissue with EDTA in the cold produced an increase in Rb^{86} uptake, most of which was erased by Ca+Mg resupplied by a rinse period. This result of increased Rb^{86} accumulation which can be reversed by divalent ion is similar to that reported by Tanada (24) with RNase. The EDTA pretreatment at room temperature decreased ion accumulation which was further depressed by Ca+Mg. The results can be interpreted as showing an endogenous enzymatic response to EDTA, which over a short time or with temperature-limited rates increases metabolic activity (cf. table II). Higher rates of enzymatic activity result in eventual inhibition of both respiration and ion accumulation.

TABLE XIII

EFFECT OF TEMPERATURE AND Ca+Mg ON IMPAIRMENT OF K ACCUMULATION BY EDTA

PRETREATMENT		RINSE SOLUTION	Rb^{86} ACCUMULATED
SOLUTION	TEMPERATURE		
			c/m/20 sections
Buffer	Ice	H_2O	2,285
		Ca + Mg	1,475
	Room	H_2O	2,163
		Ca + Mg	1,430
+ EDTA 2×10^{-3} M	Ice	H_2O	3,755
		Ca + Mg	1,793
	Room	H_2O	1,888
		Ca + Mg	607

Tissue was 1 cm sections of soybean root taken 0.5 to 1.5 cm from the tip. Pretreatment was for 1 hour on a shaker in 10^{-3} M potassium phosphate buffer (pH 6.0). Roots were next rinsed for 10 minutes on a shaker in water or 5×10^{-3} M $CaCl_2$ + 5×10^{-3} M Mg SO_4. Accumulation was for 2 hours in 2×10^{-3} M phosphate buffer + Rb^{86}. Exchangeable Rb^{86} was removed in phosphate buffer + 10^{-2} M $CaCl_2$.

DISCUSSION

If it is assumed that the sole action of RNase is to degrade polyribonucleotides, the results reported here and previously (8, 15, 24) provide strong evidence for the existence of RNA essential to respiration, ion accumulation and solute retention. The cytological site of this RNA is probably in the mitochondrial membranes and the limiting cytoplasmic membranes (plasmalemma and/or tonoplast). Ribonucleic acid is believed to occur in the cell membrane of yeast where it contributes to cation binding (21). The enzyme complex responsible for oxidative phosphorylation is considered to be in the mitochondrial membranes (16). Pinchot (22) has demonstrated that the polynucleotide required for oxidative phosphorylation in the bacterium *Alcaligenes faecalis* serves to bind electron transporting and phosphorylating components. An analagous binding role for RNA in mitochondria would explain the effect of RNase on mitochondrial activity and root respiration. In addition, polynucleotide in the limiting cytoplasmic membranes may bind enzymatic components essential to ion accumulation. Loss of such a structural moiety would also explain the noted leakage of solutes (table VIII).

The experiments reported here show that EDTA produces physiological responses similar to those elicited by RNase. The common responses are undoubtedly related through the common destruction of polynucleotides and/or the subsequent loss of solutes. The EDTA (and high concentrations of KCl) must act, then, to expose the RNA to endogenous RNase. This is directly demonstrated with root homogenates in table XII. Higuchi and Uemura (12) offer a similar explanation for the action of chelate ions in releasing nucleotides from yeast. It is possible that Ca and/or Mg (the principal divalent ions) are electrostatically bound to adjacent phosphate valences of RNA, and interfere with cleavage of the polymer. One equivalent of divalent ion can be bound per mole of polynucleotide phosphate (6). Substitution of monovalent K for the divalent ions would then permit degradative metabolism of the RNA. The application of potassium-EDTA would be particularly effective as noted here.

It is of interest in this connection that K will accelerate auxin-induced growth (5, 25), and low concentrations of EDTA will promote root growth (19, 29) and coleoptile elongation (10). (High concentrations of EDTA are growth inhibitory (19)). Perhaps some metabolism of RNA essential to growth is normally controlled by K/Ca + Mg ratio. Increasing the ratio by adding K or by chelation of divalent ions would favour RNA metabolism. Such a metabolism in roots is to be found in the decreasing amount of microsomal RNA and the increasing amount of RNA associated with mitochondrial and membranous material during cell growth (18). Part of this latter RNA may be that which is essential to respiration and ion accumulation, and may contribute to the increase in respiration and ion accumulation per unit protein known to occur with root cell expansion (3, 4, 14, 18, 28). In developing pea leaf tissue, Smillie & Krotkov (23) have described a correlation between RNA and photosynthetic and respiratory energy producing mechanisms.

The experiments with successive 0.5 cm root sections were undertaken to determine if changes in cell size and maturity would be reflected in altered sensitivity to EDTA. Although the affect of EDTA varies with cell maturity, no single pattern of change is evident. Previous observations of an increase in respiration and ion accumulation with cell growth are confirmed (respiration and ion accumulation, table XI, divided by protein content, tableXII). There is an increase in RNase activity with growth; the maximum activity occurs in the second section (0.5-1.0 cm) where the loss of soluble nucleotides and the impairment of respiration and K absorption are maximal. The loss of polynucleotides in this section is quite large. This correlation between loss of physiological function and RNase activity supports the hypothesis outlined above: that is, part of the RNA of the cell is functional with membrane-associated enzymes which carry out respiration and ion accumulation. Loss of protective divalent ions leads to hydrolysis of this RNA by endogenous RNase, with subsequent impairment of physiological function. The hypothesis, however, is not compatible with the maximum impairment of phosphate uptake and ion retention in the tip section where RNase activity is minimal.

In this respect it must be recognized that the removal of divalent cations by chelation (or by displacement with high concentrations of K) could impair root activity in other ways than by induction of RNA degradation. The loss of cations essential to oxidative metabolism could reduce respiration. Honda (13) has described such an effect in ascorbate oxidation by barley roots, but he did not report that EDTA, which he used as a buffer, impaired endogenous respiration. The loss of acid soluble nucleotides with EDTA, RNase or high concentrations of KCl is quite marked; it is likely that the loss of adenine nucleotides and coenzymes is a factor in the decline in respiration. However, the impaired activity of mitochondria isolated from EDTA treated roots (table VII) shows that some change beyond the loss of dissociable cofactors is involved; Mg, AMP, and dissociable respiratory cofactors were supplied to these mitochondria. Furthermore, direct application of RNase to mitochondria produces a similar impairment of activity (8).

Ion accumulation is dependent upon aerobic respiration and semi-permeable membranes. It is not clear in these experiments whether the diminished ion accumulation which follows EDTA application is due to degradation of ion accumulation sites, diminished respiration, "leaky" membranes, or all of these. Again it is possible that chelation of divalent cations has some direct effect on ion uptake and retention. The loss of Mg might well be of primary importance in phosphate accumulation since the process is believed related to oxidative phosphorylation (7), where Mg is required. Helder (11) has reported a leakage of Rb^{86} from barley roots in the absence of exogenous Ca or in the presence of sulphate ion. (The effect of the sulphate could be through the removal of Ca as the insoluble salt.) It is possible that the simple adsorption of Ca to the membrane serves to maintain semi-permeability. Helder's experiments, however, may have also involved degradation of RNA induced by removal of the Ca. Certainly the effect of RNase in causing a leakage of accumulated ion (table VIII) similar to that induced by EDTA suggests that the basic cause of leakage lies in RNA degradation. The temperature dependence of the EDTA effect on K accumulation (table XIII) implies that an enzymatic process is induced by the chelation of Ca and Mg.

The transitory respiratory burst which is obtained shortly after applying RNase or EDTA to the root tissue is not readily explained. The same effect can be obtained by applying RNase to mitochondria (8), but it is accompanied by lowered phosphorylation. Evidently, a partial hydrolysis of the functional RNA leads to an uncoupling of phosphorylation. It is assumed here that EDTA produces the same effect in vivo by induction of RNA degradation. The respiratory burst might also be explained by additional adenine nucleotides being made available, or by mitochondrial membranes becoming more permeable to substrate penetration, but not yet irreparably damaged.

The increased respiration found with K phosphate or chloride (salt-respiration) could be related to that induced by EDTA or RNase. Substitution of K for some divalent ion could permit a partial degradation of mitochondrial RNA by endogenous RNase, leading in turn to the respiratory increase as discussed above. Of course, if the K concentration were too high, and too much divalent ion were substituted, the RNA degradation would ultimately lead to respiratory inhibition (table III).

Summary

Treatment of soybean or corn root tips with ribonuclease, ethylenediaminetetraacetic acid or high concentrations of KCl impaired respiration after one to three hours. Concurrently, cold-acid soluble nucleotides were lost, partly to the medium; polynucleotides

began to degrade. The lowered respiration was related to loss of mitochondrial activity. Cell membranes lost their semi-permeability. The expanding root zone showed the greatest loss of soluble nucleotides and polynucleotides, respiration and K accumulation when treated with EDTA. This zone had the highest RNase activity. Phosphate accumulation and ion retention were most impaired in the meristematic and early cell expansion zones.

It is believed that RNA in membranes is implicated in ion accumulation, solute retention, and oxidative phosphorylation. It is thought that divalent ions normally protect this RNA from degradation by endogenous enzymes. Substitution of monovalent ions for divalent ions leads to the degradation of membranous RNA and the loss of physiological and biochemical functions. Normal metabolism of RNA could be governed by K/Ca + Mg ratios. Salt respiration may result from partial degradation of mitochondrial RNA.

Acknowledgement

The author is indebted to Mrs. Mary Fisher for technical assistance.

Literature Cited

1. Brachet, J. 1954. Effects of ribonuclease on the metabolism of living root tip cells. Nature 174: 876–877.
2. Brachet, J. 1955. Further observations on the effects of ribonuclease on living root-tip cells. Biochem. Biophys. Acta 16: 611–612.
3. Brown, R. and D. Broadbent. 1950. The development of cells in the growing zones of the root. Jour. Exp. Bot. 1: 249–263.
4. Brown, R. and P. M. Cartwright. 1953. The absorption of potassium by cells in the apex of the root. Jour. Exp. Bot. 4: 197–221.
5. Cooil, B. J. 1952. Relationships of certain nutrients, metabolites, and inhibitors to growth in the *Avena coleoptile*. Plant Physiol. 27: 49–69.
6. Felsenfeld, G. and S. Huang. 1959. The interaction of polynucleotides with cations. Biochem. Biophys. Acta 34: 234–242.
7. Hagan, C. E., Jr., J. E. Leggett, and P. C. Jackson. 1957. The sites of orthophosphate uptake by barley roots. Proc. Natl. Acad. Sci., U.S. 43: 496–506.
8. Hanson, J. B. 1959. The effect of ribonuclease on oxidative phosphorylation by mitochondria. Jour. Biol. Chem. 234: 1303–1306.
9. Hanson, J. B. and J. S. Kahn. 1957. The kinetics of potassium accumulation by corn roots as a function of cell maturity. Plant Physiol. 32: 497–498.
10. Heath, O. V. S. and J. E. Clark. 1956. Chelating agents as plant growth substances: a possible clue to the action of auxin. Nature 177: 1118–1121.
11. Helder, R. J. 1958. Studies on the absorption, distribution, and release of labelled rubidium ions in young intact barley plants. Acta. Bot. Neerl. 7: 235–249.
12. Higuchi, M. and T. Uemura. 1959. Release of nucleotides from yeast cells. Nature 184: 1381–1383.
13. Honda, S. 1957. Some effects of ascorbic acid and metal complexing agents on the respiration of barley roots. Plant Physiol. 32: 23–31.
14. Kahn, J. S. and J. B. Hanson. 1957. The effect of calcium on potassium accumulation in corn and soybean roots. Plant Physiol. 32: 312–316.
15. Lansing, A. I. and T. B. Rosenthal. 1952. The relation between ribonucleic acid and ionic transport across the cell surface. Jour. Cell Comp. Physiol. 40: 337–345.
16. Lehninger, A. L., C. L. Wadkins, C. Cooper, T. M. Devlin, and J. L. Gamble. 1958. Oxidative phosphorylation. Science 128: 450–456.
17. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin-phenol reagent. Jour. Biol. Chem. 193: 265–275.
18. Lund, H. A., A. E. Vatter, and J. B. Hanson. 1958. Biochemical and cytological changes accompanying growth and differentiation in the roots of *Zea mays*. Jour. Biophys. Biochem. Cytology 4: 87–98.
19. Majumber, S. K. and S. Dunn. 1958. Effect of metal chelates on growth of corn in solution cultures. Plant Physiol. 33: 166–169.
20. Ogur, M. and G. Rosen. 1950. The extraction and estimation of desoxypentose nucleic acid and pentose nucleic acid. Arch. Biochem. 25: 262–276.
21. Passow, H., A. Rothstein, and B. Loewenstein. 1959. An all-or-none response in the release of potassium by yeast cells treated with methylene blue and other basic redox dyes. Jour. Gen. Physiol. 43: 97–107.
22. Pinchot, G. B. 1959. A polynucleotide coenzyme of oxidative phosphorylation. III. Mechanism of action. Biochem. Biophys. Res. Communications 1: 17–20.
23. Smillie, R. M. and G. Krotkov. 1959. Ribonucleic acid as an index of the metabolic activity of pea leaves. Biochem. Biophys. Acta. 35: 550–551.
24. Tanada, T. 1956. Effect of ribonuclease on salt absorption by excised mung bean roots. Plant Physiol. 31: 251–253.
25. Thimman, K. V. and C. L. Schneider. 1938. The role of salts, hydrogen-ion concentration and agar in the response of the *Avena coleoptile* to auxins. Amer. Jour. Bot. 25: 270–280.
26. Ts'o, P. O. P. 1958. Structure of microsomal nucleoprotein particles from pea seedlings. In: Microsomal Particles and Protein Synthesis. Pp. 156–168. Symposium Biophys. Soc., Washington Acad. Sci., Washington, D. C.
27. Umbreit, W. W., R. H. Burris, and J. F. Stauffer. 1957. Manometric Techniques, 3rd Ed. Burgess Publishing Co., Minneapolis.
28. Wanner, H. 1950. Histologishe und physiologishe gradienten in der wurzelspitze. Ber. schweiz. bot. Ges. 60: 404–412.
29. Weinstein, L. H., A. M. Meiss, R. L. Uhler, and E. R. Purvis. 1956. Effect of ethylenediaminetetraacetic acid on nitrogen metabolism and enzyme patterns in soybean plants. Cont. Boyce Thompson Inst. 18: 357–370.
30. West, S. H. 1958. Ph.D. dissertation, University of Illinois.

(2-CHLOROETHYL)TRIMETHYLAMMONIUM CHLORIDE AND RELATED COMPOUNDS AS PLANT GROWTH SUBSTANCES
II. EFFECT ON GROWTH OF WHEAT[1,2]

N. E. TOLBERT

DEPARTMENT OF AGRICULTURAL CHEMISTRY, MICHIGAN STATE UNIVERSITY, EAST LANSING

In this series of publications, the action of (2-chloroethyl)trimethylammonium chloride and certain other compounds structurally related to the quaternary ammonium cation, $(CH_3)_3N^+CH_2\text{-}CH_2X$, are described as a class of plant growth substances. In the first paper (1), compounds were examined for biological activity by an assay with wheat seedlings which involved the measurement of the distance between the bases of the first two leaf blades. Three very active and closely related compounds were discovered in which X was a chloro, bromo or $=CH_2$ group, namely, (2-chloroethyl)trimethylammonium chloride, (2-bromoethyl)trimethylammonium bromide and (2,3-*n*-propylene)trimethylammonium bromide.[3]

The growth of wheat plants after various treatments with the three most active derivatives is described in this paper. The most characteristic growth change was a reduction in height of plants accompanied by an increase in stem diameter. In most respects the growth alterations after treatment with derivatives related to (2-chloroethyl)trimethylammonium chloride were the opposite from that obtained with gibberellin, and in addition, these effects were reversed by gibberellin. In fact, the actions of gibberellin and (2-chloroethyl)trimethylammonium chloride on the growth of plants have been found to be mutually antagonistic.

MATERIALS AND METHODS

The three quaternary ammonium salts used in the present investigation were synthesized by the reaction of trimethylamine with the corresponding dihalide, i.e., 1,2-dibromoethane for preparing (2-bromoethyl)-trimethylammonium bromide (1).

For routine experiments 15 to 18 wheat seedlings (*Triticum vulgare* of variety Thatcher unless otherwise specified) were grown in 8 in. pots with a sand and loam soil mixture. Greenhouse temperatures were 56° F ± 3 at night and 60° to 70° F during the day except as indicated otherwise. All data were obtained from experiments which were conducted during the winter and spring when light intensity in the greenhouse was low. Supplementary light from 500 watt incandescent bulbs extended the day length to 16 hours. Since the chemicals were neutral salts, aqueous solutions were used in the tests. Seedling plants were treated once at 11 days after planting the seed, at which time the second leaf was emerging. For soil treatments, 500 ml of a designated molarity of chemical was poured onto the soil. For spray treatments, solutions containing about 0.01 % aerosol OT as wetting agent were sprayed onto the leaves of the seedlings until the leaves were wet. Seed treatment consisted of placing seeds overnight on filter paper moistened with a solution of the chemical and then planting them immediately in untreated soil. Gibberellin was applied only by spray treatment to the leaves of seedling plants.

Two weeks after the soil or spray treatment, the distance from the base of the first leaf blade to the base of the second leaf blade for each plant was measured in millimeters. The data are average values from measurements on at least 15 plants. The distance between the second and third leaf blades at the time of the first measurement was rapidly increasing, and thus, this length was not consistent even in the controls. Therefore, four weeks after treatment, the total stem height of the plants from the soil to the base of the uppermost leaf blade was also recorded in some experiments.

Plants from a few treatments were kept to maturity to observe later growth patterns and yield of grain. In these cases, 12 in pots were used and Hoagland nutrient solution was applied about every ten days.

RESULTS AND DISCUSSION

TREATMENTS AND GENERAL EFFECT ON GROWTH: When wheat plants were treated once with either (2-chloroethyl)trimethylammonium chloride, (2-bromoethyl)trimethylammonium bromide or (2,3-*n*-propylene)trimethylammonium chloride or bromide, the major growth difference was the development of

[1] Received August 19, 1959.

[2] Published with approval of the Director of the Michigan Agricultural Experiment Station as Journal Article No. 2478.

[3] In the literature these compounds have also been referred to as derivatives of choline. Thus, (2-bromoethyl)-trimethylammonium bromide has been called bromocholine bromide and (2-chloroethyl)trimethylammonium chloride would be called chlorocholine chloride.

TABLE I

LENGTH OF THATCHER WHEAT PLANTS AFTER TREATMENT WITH (2-CHLOROETHYL)TRIMETHYLAMMONIUM CHLORIDE OR (2-BROMOETHYL)TRIMETHYLAMMONIUM BROMIDE*

TREATMENT	2 WEEKS MM	4 WEEKS MM
None	34	260
Soil application of (2-chloroethyl)trimethylammonium chloride		
10^{-2} M	−3	78
10^{-3} M	1	91
10^{-4} M	7	139
10^{-5} M	15	153
10^{-6} M	23	200
Soil application of (2-bromoethyl)trimethylammonium bromide		
10^{-3} M	5	75
10^{-4} M	9	131
10^{-5} M	13	171
10^{-6} M	18	188
Leaf spray of (2-bromoethyl)trimethylammonium bromide		
10^{-2} M	6	...
10^{-3} M	9	...
5×10^{-4} M	14	...

* The measurements at 2 weeks after treatment were the distance between the bases of the first and second leaf blades. The values at 4 weeks after treatment represented the total stem length to the base of the uppermost leaf. Values are the average from 15 to 18 plants.

plants with shorter and thicker stems than in untreated plants (all figures and tables) and the leaves were of darker green color. Growth effects from the three compounds were the same and they were active over the same range of concentrations. Most of the experiments were repeated with all three of the compounds, but only one chemical name, (2-chloroethyl)-trimethylammonium chloride, is used in this manuscript to represent this group of three compounds.

Soil treatment of seedlings was effective after one application of 10^{-2} to 10^{-6} M solutions (table I). For (2-chloroethyl)trimethylammonium chloride, a 10^{-6} M solution is equivalent to 0.13 ppm. From one application of a 10^{-4} to 10^{-6} M solution to the soil 11 days after planting the seed, the distances between the bases of all leaf blades of Thatcher wheat plants were substantially reduced in length through their early vegetative growth cycle, but not at the time of heading. Corresponding to the decrease in stem length, the width of stems from treated plants was thicker than stems on control plants. The shorter and thicker stems resulted in wheat plants which grew very erect with no tendencies toward lodging.

Earlier treatments than used in the spray and soil experiments reported here caused the stems to be shorter than recorded in the tables. Solutions of 10^{-3} M or stronger reduced the first interleaf distance essentially to zero. Applications of 10^{-2} M solutions so reduced the elongated growth of the plant that the base of the second leaf remained below the base of the first leaf as indicated by a negative value in the table. As a result, the base of the second leaf grew out through the sheath of the older first leaf. At 10^{-2} M concentration total growth of the plants as measured by weight was at first reduced but there were no other toxicity symptoms.

The compounds could also be applied as a spray to the leaves (table I), in which case 10^{-2} M solutions were not toxic and the lowest concentration for effectiveness was in the range of 10^{-5} M. One soil application at the same molarity was more effective than one spray treatment, probably because large volumes of dilute solutions could be applied to the soil as a more continuous source.

After any of the chemical treatments, including 10^{-2} M solutions, and during the final growth of plants at the time of heading, the upper portion of the stems elongated to produce a plant of height nearer the untreated controls. Nevertheless, mature plants which had been treated once as seedlings with greater than 10^{-5} M concentrations of the compounds remained shorter than control plants.

Treated plants were by visual inspection darker green in color, and (2,3-*n*-propylene)trimethylammonium bromide was very effective in producing this response. Detailed results concerning the increase in chlorophyll content of leaves from a variety of treated plants will be presented in another paper. With wheat plants the stockier and compact appearance of the plants also gave the impression of greener plants.

The leaves of treated plants were also shorter in length and broader in width (fig 1). In a series of measurements 3½ weeks after one treatment of plants with 10^{-3} M (2-bromoethyl)trimethylammonium bromide, the last fully developed leaf of treated plants averaged more than 9 mm in width, while the same leaf on the control plants was 7 mm wide. This growth pattern in the leaves was consistent with the development of shorter and thicker stems. This measurement of leaves was not used for routine assay because of wide biological variation among plants and the fact that the numerical values were not as greatly different as those values on stem length.

SEED TREATMENT: Seeds were placed overnight on filter paper moistened with an aqueous solution of (2-chloroethyl)trimethylammonium chloride and then planted the next morning in untreated soil. Plants were grown at day temperatures of 75° to 85° F and in high light intensities obtained in May. The appearance of these plants was similar to that obtained from treatment of seedlings. The height and wet weight of the tops after 26 days are given in table II. The results indicate that seed treatment is an effective method for applying the chemical.

TILLERING: There was a pronounced and earlier tillering of the young wheat plants (table III) within a few days to two weeks after treatment with the chemical compounds. In greenhouse experiments in the winter, the control plants developed tillers near the time of heading, and as a result, the tillers had an

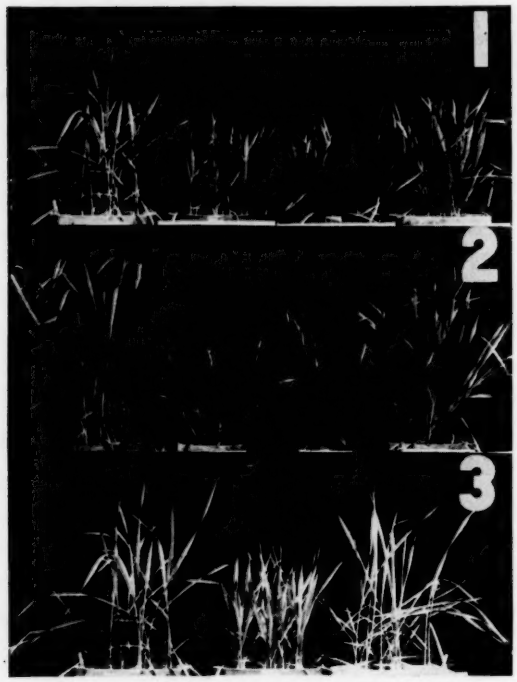


FIG. 1. Thatcher wheat seedlings 2 weeks after one soil treatment with (2-chloroethyl)trimethylammonium chloride as described in the text: from left to right, no treatment, 10^{-3}, 10^{-4}, and 10^{-5} M.

FIG. 2. Thatcher wheat 4 weeks after the same treatment as in figure 1.

FIG. 3. Combined effect of (2-bromoethyl)trimethylammonium bromide and gibberellin on Thatcher wheat. 10^{-3} M (2-Bromoethyl)trimethylammonium bromide was applied once to the soil of the two pots on the right 11 days after planting. Plants in the pot on the far right were sprayed with 100 ppm gibberellin 8 days later, and this photograph was taken after another 6 days or 14 days after the first treatment.

TABLE II

HEIGHT AND WEIGHT OF WHEAT AFTER SEED TREATMENT WITH (2-CHLOROETHYL)TRIMETHYLAMMONIUM CHLORIDE*

TREATMENT	1ST TO 2ND LEAF MM	TOTAL HEIGHT** MM	WET WT/ PLANT GM
0	19	72	0.7
10^{-1} M	7	42	0.8
10^{-2} M	8	48	0.8
10^{-3} M	11	52	0.7

* The method of treatment is described in the text. Measurements were made 26 days after planting the seed.
** From the soil to the base of the uppermost leaf.

appearance of a resurgent growth. In the treated plants almost all the tillers appeared shortly after treatment and grew almost as rapidly as the main culm, while there was correspondingly little or no tillering later during heading. This early tillering by treated plants was part of a growth pattern contributing to the appearance of bushier plants than the controls. Thus, the appearance of there being more plants in the treated pots (figure 1) was caused by the early tillering. The number of plants in each pot was the same, but the taller control plants without tillers appeared more thinly planted.

UNIFORMITY OF GROWTH: The number of leaves was not altered by the chemical treatments and the time of appearance of the early leaves was not appreciably delayed. Later leaves generally appeared 1 to 3 days after the corresponding leaf on the control plants. The treated plants were much more uniform in height than control plants (fig 1) and in time of heading of the grain. This uniformity in height of treated plants was accentuated by the fact that the tillers in the treated plants developed at nearly the same rate as the first stalk rather than developing later.

RAPIDITY AND PERSISTENCE OF EFFECT: The visual changes in the growth of wheat after one treatment with these compounds developed slowly over a period of two weeks. A difference in stem length between controls and treated plants could first be detected after three to five days. The persistence of the effects from one application of the compounds for weeks afterwards suggested that these substances were not rapidly destroyed in the plant and were translocated into the new leaves.

WET WEIGHT, MATURATION, AND CROP YIELD: When low concentrations of (2-bromoethyl)trimethylammonium bromide were applied to the soil, there were no differences in wet or dry weight of the tops of Thatcher or Russell wheat plants 40 days after planting as compared with the controls, even though the treated plants were shorter in height. The wet weight of young seedlings also was not altered during growth after prior seed treatment, even though the growth pattern was strikingly different (table II).

Experiments on maturation of treated wheat plants were conducted in the greenhouse, but the results were only suggestive because of restrictions on growth induced by the pot and by insufficient light. There was no decrease in yield from treatments sufficient to reduce height of the plant, and in fact, some of the greenhouse experiments indicated an increase in yield by plants treated with 10^{-5} to 10^{-6} M solutions. This increase was accounted for by a higher weight per kernel of grain, since the total number of kernels per head and the total number of heads of grain per plant did not vary. All these experiments on growth suggested that the action of the chemical was to alter the developmental pattern rather than rate of growth, and thus the compounds have been called growth substances.

TABLE III

EFFECT ON TILLERING*

TREATMENT	NO. OF TILLERS/NO. OF PLANTS**
0	3/16, 7/15, 3/16
10^{-3} M (2-chloroethyl)trimethylammonium chloride	20/16
10^{-4} M (2-chloroethyl)trimethylammonium chloride	20/18
10^{-3} M (2,3-*n*-propylene)trimethylammonium bromide	23/16
10^{-4} M (2,3-*n*-propylene)trimethylammonium bromide	19/16
10^{-3} M (2-bromoethyl)trimethylammonium bromide	21/18, 14/15
10^{-3} M (2-bromoethyl)trimethylammonium bromide + 100 ppm gibberellin***	1/17, 1/16
10^{-3} M (2-bromoethyl)trimethylammonium bromide + 100 ppm gibberellin†	11/16

* Tillers were counted 3 weeks after treatment with chemicals which were applied as described in the text.
** Different results for the same treatment were from different pots of plants.
*** 100 ppm gibberellin was sprayed on the plants either at the time of application of (2-bromoethyl)trimethylammonium bromide or up to eight days later with the same results.
† These plants were sprayed with gibberellin 14 days after the treatment with (2-bromoethyl)trimethylammonium bromide.

Treatments with these compounds also caused a delay in the time of final ripening of wheat plants in the greenhouse for at least two reasons. The control plants headed two to four days before the treated plants. In addition, during a two-week period after heading, when the wheat kernels were maturing, the treated plants remained greener than the control plants which slowly turned yellow. Thus, there was in greenhouse experiments an extended period during which the treated plant appeared more capable of furnishing the ripening seed extra carbohydrates.

TEMPERATURES: In one series of experiments wheat plants were grown under three different conditions of temperatures: A. 65° to 70° F during the day and 55° to 58° F at night; B. 70° to 75° F during the day and 65° to 68° at night; C. about 85° F and above during the day and about 80° F at night. For good growth of wheat it is usually considered that night temperatures of 55° F or lower are necessary. When applied by soil treatment to plants at all three temperatures, (2-bromoethyl)trimethylammonium bromide had the same effect of decreasing the distance between the leaf bases, though the growth of the plants at the highest temperature was poor. The results suggest the possibility that the compounds may be beneficial to some plants by preventing severe elongation from the effect of high night temperatures. Although the compounds were most effective near the optimum growth temperature, they may be useful when applied at high temperatures where satisfactory growth of wheat cannot normally be obtained.

LIGHT INTENSITY: The growth pattern obtained after treatment with (2-chloroethyl)trimethylammonium chloride is, in general, characteristic of growth in high intensity light. In this respect, these chemical compounds reversed the elongated growth pattern obtained in low light intensity. In total darkness or light intensities of about 10 ft-c, however, the compounds were without effect.

All the experiments reported in this paper were performed from November through February, during which months light intensity and total light obtained in Michigan were low. The compounds were retested during April when the light intensity in the greenhouse was much higher, and the conclusions were the same. At the higher light intensity obtained in April, however, the magnitude of the effect with the same concentration of the chemicals was somewhat less, since the distance between the bases of the first leaf blades of the control plants was less in high light intensity.

OTHER GRAINS: Since the chemicals were only tested extensively with Thatcher wheat, a spring wheat, a few other grains were treated. (2-Bromoethyl)trimethylammonium bromide was about equally effective on another spring wheat, Russell and the chemical was active also on Genesee, a winter wheat. (2-Bromoethyl)trimethylammonium bromide caused Montcalm barley and Gary oats to grow with shorter internodes (table IV), but the effects were less than on spring wheats. The treated barley plants in the greenhouse were sturdier and greener than the untreated controls.

COMBINED TREATMENTS OF (2-CHLOROETHYL)TRIMETHYLAMMONIUM CHLORIDE AND GIBBERELLIN:

TABLE IV

DISTANCE BETWEEN BASES OF 1ST AND 2ND LEAF BLADES OF BARLEY AND OATS AFTER TREATMENT WITH (2-BROMOETHYL)TRIMETHYLAMMONIUM BROMIDE*

TREATMENT	MONTCALM BARLEY MM	GARY OATS MM
0	50	42
10^{-2} M	23	21
10^{-3} M	29	32

* Measurements were made 2 weeks after treatment of 11 day old seedlings.

The plant growth promoting action of the (2-chloroethyl)trimethylammonium chloride type of compound seemed to be by inspection opposite to the action of gibberellin. Gibberellin promoted stem elongation and the development of spindly plants, while (2-chloroethyl)trimethylammonium chloride produced shorter stems and stockier plants. Both classes of compounds by themselves were effective at low concentrations, though (2-chloroethyl)trimethylammonium chloride was active at as low as 0.1 ppm on the wheat, whereas 10 ppm of gibberellin was necessary to cause an appreciable effect by itself. After gibberellin treatment, leaves were lighter green than controls; after (2-chloroethyl)trimethylammonium chloride, the leaves were darker green. Certain combinations of the two chemical treatments produced plant growth of nearly normal appearance since the effects from one chemical were nullified by the other.

When excess gibberellin and (2-chloroethyl)trimethylammonium chloride were applied simultaneously to young Thatcher wheat seedlings, the action of gibberellin completely dominated so that there was no decrease in the height of the plants which had been treated with both compounds compared to plants treated with gibberellin only (table V, experiment A). Therefore, lower concentrations of gibberellin with excess (2-chloroethyl)trimethylammonium chloride were tried. At 10 ppm of gibberellin, 10^{-2} M (2-chloroethyl)trimethylammonium chloride prevented most of the elongation obtained from the gibberellin treatment. Similar results were obtained with 10 ppm gibberellin and 10^{-4} M solutions of the compounds. The action of gibberellin was particularly effective for increasing the stem length between the bases of the first two leaf blades, and (2-chloroethyl)-trimethylammonium chloride was only partially effective in preventing this elongation. At 10 ppm or less of gibberellin the action of (2-chloroethyl)trimethylammonium chloride dominated after about one to two weeks, so that the distance between the bases of the second and third leaf blades was very short. These results indicate that (2-chloroethyl)trimethylammonium chloride prevented or inhibited the growth expression from 10 ppm gibberellin. The results also illustrate loss of the gibberellin effect a few days after treatment and the persistence of one treatment of (2-chloroethyl)trimethylammonium chloride. One ppm of gibberellin by itself was without visible effect on the wheat, but 1 ppm of gibberellin was still able to reverse a substantial portion of the effect from excess (2-chloroethyl)trimethylammonium chloride.

Gibberellin reversed the effects of a (2-chloroethyl)trimethylammonium chloride treatment many days after the treatment with the 2-chloroethyl derivative (table V, experiment B and fig 3). Eight days after treatment with (2-chloroethyl)trimethylammonium chloride alone, short plants had developed as illustrated by the middle pot in figure 3. These plants

TABLE V

COMBINED APPLICATIONS OF (2-CHLOROETHYL)TRIMETHYLAMMONIUM CHLORIDE AND GIBBERELLIN TO THATCHER WHEAT SEEDLINGS

TREATMENT	DISTANCE BETWEEN LEAF BASES AFTER 2 WKS		TOTAL STEM HEIGHT AFTER 4 WKS
	1ST-2ND	2ND-3RD	
	MM	MM	MM
Exp. A (simultaneous treatments)			
None	37	43	
100 ppm gibberellin	58	49	
100 ppm gibberellin + 10^{-2} M (2-chloroethyl)trimethylammonium chloride	60	44	
10 ppm gibberellin	48	47	
10 ppm gibberellin + 10^{-2} M (2-chloroethyl)trimethylammonium chloride	37	4	
1 ppm gibberellin	38	43	
1 ppm gibberellin + 10^{-2} M (2-chloroethyl)trimethylammonium chloride	18	7	
10^{-2} M (2-chloroethyl)trimethylammonium chloride	3	0*	
Exp. B			
None	34	32	260
10^{-3} M (2-bromoethyl)trimethylammonium bromide	5	0*	112
10^{-3} M (2-bromoethyl)trimethylammonium bromide + 100 ppm gibberellin simultaneously	55	24	258
10^{-3} M (2-bromoethyl)trimethylammonium bromide + 100 ppm gibberellin 3 days later	18	36	244
10^{-3} M (2-bromoethyl)trimethylammonium bromide + 100 ppm gibberellin 14 days later	5	0*	269

* The third leaf was present but the leaf base had not emerged from the sheath of the second leaf.

quickly and completely reverted to a normal or elongated plant after one application of gibberellin. When gibberellin was applied, elongation occurred most between the bases of leaf blades which were then forming, but also renewed growth took place all along the wheat stem if heading had not occurred.

At the time of heading of wheat which had been treated only with (2-chloroethyl)trimethylammonium chloride, elongation of the plants occurred so that the final height of the heads of grain was near that of the controls. It is possible that the production of natural gibberellin-like material during heading reversed the effect of the (2-chloroethyl)trimethylammonium chloride.

Experiments on the mutual antagonistic effect of gibberellin and (2-chloroethyl)trimethylammonium chloride on the growth of plants have not been explored in crop production. In greenhouse experiments, wheat, which had been treated with both gibberellin and (2-cholorethyl)trimethylammonium chloride so that growth in height was about normal, appeared greener and stouter than the untreated plants. Another possibility would be to initiate earlier lateral development by treatment with (2-chloroethyl)trimethylammonium chloride, and then with gibberellin stimulate normal or tall growth of both the main plant and the lateral developments. Early tillering produced by (2-chloroethyl)trimethylammonium chloride was prevented by simultaneous application of gibberellin (table III), but it would be impossible to reverse this effect if the gibberellin were applied after the tillers had become well established.

Summary

Compounds of the structure $(CH_3)_3N^+CH_2\text{-}CH_2X$ were active as plant growth substances when X was a Cl, Br or $=CH_2$ group. Solutions of (2-chloroethyl)trimethylammonium chloride, (2-bromoethyl)-trimethylammonium bromide and (2,3-*n*-propylene)-trimethylammonium bromide or chloride were effective from 10^{-2} to 10^{-6} M when poured onto the soil or used in nutrient culture. Though the chemicals were most effective when applied to the soil, they could also be sprayed on the leaves, or added by soaking of seeds before planting. One treatment was effective on the total growth of the plant. The most characteristic growth alterations were shorter and thicker stems, broader and greener leaves, earlier and stronger tillering, and more uniform growth. These alterations in growth occurred without a change in wet or dry weight. The growth changes were similar to those produced by high light intensity and the opposite from those caused by gibberellin. Gibberellin reversed the action of these compounds on growth and they reversed the effect of gibberellin.

Acknowledgements

I am indebted to Dr. E. Everson and Dr. J. E. Grafius for greenhouse assistance and advice. I appreciate the encouragement and advice from Dr. S. H. Wittwer during the development of this project; with him we are extending the study to other plants and other aspects of growth.

Literature Cited

1. Tolbert, N. E. 1960. (2-Chloroethyl)trimethylammonium chloride and related compounds as plant growth substances. I. Chemical structure and bioassay. Jour. Biol. Chem. 235: 475–479.

FORMATE OXIDATION BY PARTICULATE PREPARATIONS FROM HIGHER PLANTS[1]

MENDEL MAZELIS

WESTERN REGIONAL RESEARCH LABORATORY,[2] ALBANY, CALIFORNIA

Enzyme systems capable of oxidizing formate to CO_2 are of wide occurrence in higher plants (3). Seed extracts have been used primarily as the source of enzyme. Extracts of rat liver and kidney were also able to carry out this oxidation (9). The enzyme from leguminous seeds, such as pea and bean, has been studied in considerable detail (1, 3, 4, 9). The formic dehydrogenase activity in various organs of the pea has been followed throughout the life cycle (3). The highest activity was found in the mature seed. This activity diminished upon germination and growth. Seeds of 93 species were tested and 54 were shown to be capable of oxidizing formate (3). The intracellular localization of the enzyme has not been reported for either plant or animal tissues.

The present study reports the presence of a formate-oxidizing system in cabbage leaf localized in the cytoplasmic particles sedimenting between $1000 \times G$ and $23{,}000 \times G$ during centrifugation. Characteristics of this enzyme have been examined in some detail and compared with the classical pea seed dehydrogenase. The latter has also been found in the present study to be mainly in the particulate fraction. The enzyme is even more widely distributed than previously believed, since active preparations have been obtained from plants which had given negative results with seed extracts. Among these are cabbage and several other members of the Cruciferae. In every case the activity is localized in the particulate fraction obtained by centrifugation.

EXPERIMENTAL METHODS

PREPARATION OF ENZYME: All plant sources were purchased at local markets. Washed cytoplasmic particle suspensions were prepared as described previously (11), with slight modifications depending upon the material. Soluble cytoplasmic proteins of pea and cabbage were precipitated from the supernatant fluid obtained after centrifugation at $23{,}000 \times G$. This solution was made 75 % saturated with solid $(NH_4)_2SO_4$ at 4° C. After 2 hours in the cold, the precipitate was collected by centrifugation, dissolved in distilled water, and dialyzed against several changes of 0.01 M Na_2HPO_4 overnight (4). Insoluble material was centrifuged down and discarded, and the solution was lyophilized. An acetone powder of cabbage particles was made from a preparation which had been suspended in and then dialyzed against 0.01 M phosphate at pH 7.0 for 60 to 90 minutes to remove any remaining sucrose. Four volumes of −20° C. acetone were added slowly with stirring; the mixture was kept at ice temperature for 30 minutes, filtered, washed, and dried in vacuo in the cold. A 1 % suspension of the acetone powder was made in 0.01 M phosphate pH 7.0. After 20 minutes in the cold, it was centrifuged and the clear solution used as the enzyme source. A digitonin extract of cabbage particles was prepared by adding 2 volumes of a 1 % digitonin suspension and shaking occasionally for 10 minutes at 4° C. The insoluble material was removed by centrifugation. The solution was made 75 % saturated with solid $(NH_4)_2SO_4$. The precipitate was collected by centrifugation and dissolved in a small volume of 0.01 M phosphate pH 7.0, and dialyzed several hours against the same buffer. Some material precipitated within the dialysis sack and was discarded. The clear dialysate was used as the enzyme.

STANDARD ASSAY PROCEDURES: The reaction was carried out in conventional Warburg vessels with continuous shaking at 30° C. The activity was determined by the conversion of $HC^{14}OONa$ to $C^{14}O_2$, which was trapped by absorption in 0.2 ml 20 % KOH in the center well. The reaction was terminated by tipping in 0.3 ml $5N$ H_2SO_4 from the sidearm (10 % trichloracetic acid was used on occasion). After shaking at least 10 minutes more, the contents of the center well were collected, the well washed with distilled water several times, and the washings combined with the KOH. The absorbed $C^{14}O_2$ was precipitated as $BaCO_3$, plated, and counted as described by Stumpf (13). Protein was determined by the Biuret method as described by Weichselbaum (17). After heating at 37° C for 30 minutes, the samples were cooled to room temperature and read by means of an Evelyn colorimeter at 540 mμ. Armour crystalline bovine plasma albumin was used as the standard. The Ag-Ba double salt of phosphoenolpyruvate (PEP), and the barium salt of phosphoglyceric acid (PGA), were converted to the Na salt before use.

RESULTS

LOCALIZATION OF ENZYME IN CABBAGE LEAF: The formate-oxidizing system was almost completely localized in the particulate fraction isolated from cab-

[1] Received August 24, 1959.

[2] A laboratory of the Western Utilization Research and Development Division, Agricultural Research Service, U. S. Department of Agriculture.

TABLE I

INTRACELLULAR DISTRIBUTION OF CABBAGE LEAF FORMATE-OXIDIZING SYSTEM

REACTION SYSTEM	TOTAL COUNTS/MG PROTEIN	
	PARTICLES	CYTOPLASM
Complete	7,450	126
Minus DPN	6,680	28
Minus DPN + ATP	4,050	31
+ ATP	4,350	556
Heated complete	8	...

The complete reaction mixture contained 100 μmoles phosphate pH 7.0; 1 μmole Na formate-C^{14} (120,000 total counts); 0.2 μmole DPN; 20 μmoles GSH; enzyme and distilled water to a final volume of 2 ml. 10 μmoles neutralized Na ATP were added where indicated. An aliquot of the washed particle suspension was inactivated by heating in a boiling water bath for 5 minutes for use in the heat-treated system. The incubation time was 60 minutes. 9.7 and 7.7 mg protein were present in the particulate and cytoplasmic experiments, respectively.

bage leaf homogenates (table I). Adding DPN to the washed particles resulted in a slight stimulation. The activity of the lyophilized cytoplasmic proteins increased 4.5 times on addition of DPN, but this was still only 2 % as active as the particles. Cabbage cytoplasm has a very active nucleotide pyrophosphatase (11). On adding ATP, which would tend to protect the DPN, the cytoplasmic activity was raised another four times, but this was still only 8 % of the activity exhibited by the particles. Interestingly enough, adding ATP to the particulate system resulted in a marked inhibition. The lack of any significant stimulation of the particulate activity by DPN could mean either an enzyme different from the classical DPN-requiring formic dehydrogenase is involved, or that the particle contained sufficient bound DPN so that exogenously added coenzyme had little effect. Several experiments with freshly prepared cabbage particles showed that a 10 to 20 % stimulation was usually obtained by adding DPN. The particles, however, are very active in its absence. Solubilization studies to be described later will demonstrate the DPN requirement much more clearly. The particulate system also showed a consistent slight stimulation on adding GSH; therefore, it was customarily added. The reaction time was 60 minutes in the experiment described in table I. Subsequent experiments were usually of 30 minutes duration since this was the period during which formate oxidation proceeded linearly, however, there was no increased effect of DPN with the shorter incubation time.

OPTIMUM PH: A sharp optimum in activity was exhibited at pH 7.0 in phosphate buffer (fig 1). This differed from the pea seed dehydrogenase which had an optimum activity at pH 6.0, decreasing gradually at more alkaline pH (3). Since the cabbage enzyme was particulate-bound, this optimum may reflect the optimum for formate permeability and not oxidation. Phosphate was much better than tris(hydroxymethyl)aminomethane or collidine buffers at the same pH. This was not the result of a phosphate requirement since tris(hydroxymethyl)aminomethane + 10 μmoles of phosphate gave no stimulation over the buffer alone.

OXIDATION AS FUNCTION OF TIME: The conversion of formate to CO_2 was approximately linear with time for the first 30 minutes. There was a leveling off of oxidation after about 50 % of the formate had been oxidized. In several duplicate runs the leveling off always came at the point where approximately half the added formate had been oxidized. Tipping in additional formate-C^{14} at this point did not increase the amount of $C^{14}O_2$ formed. This could result from a gradual inactivation of the enzyme or the DPNH-oxidizing system.

K_s OF PARTICULATE SYSTEM: A Lineweaver-Burk plot (8) is shown in figure 2. The K_s was found to be 5×10^{-3} M. Adler and Sreenivasaya (1) obtained a value of 7×10^{-3} M for the pea seed dehydrogenase.

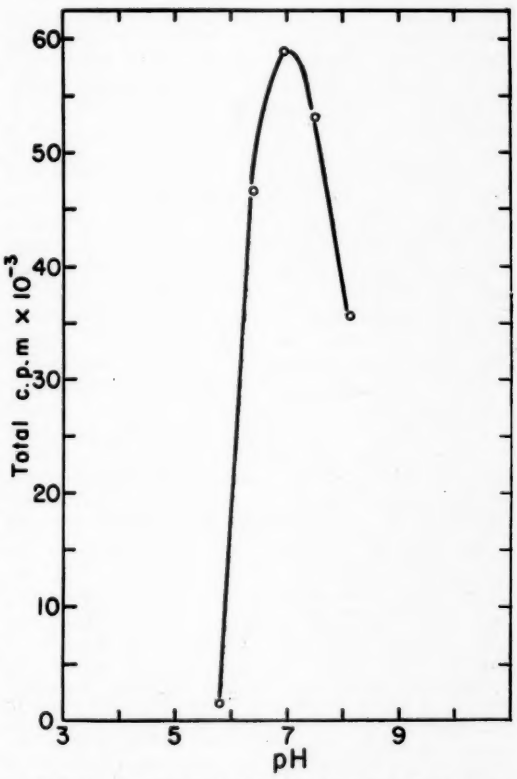


FIG. 1. Formate oxidation as a function of pH. Vessels contained the complete reaction mixture described in table I. Phosphate buffer used for whole range of pH studied. A washed particle suspension containing 10.5 mg protein was used as the enzyme source in each vessel. Incubated for 60 minutes at 30° C.

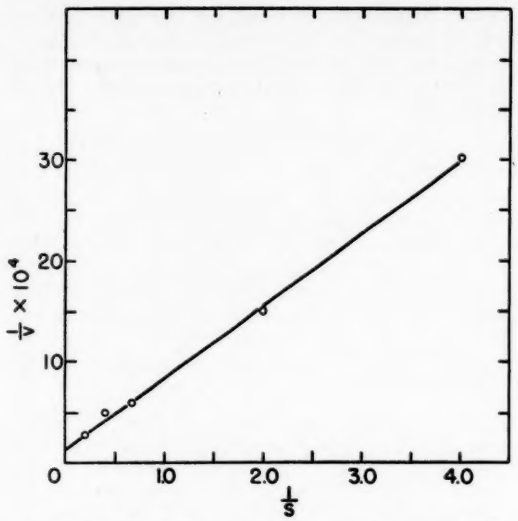


FIG. 2. Effect of substrate concentration on velocity. Na formate-C^{14} used as substrate, given in μmoles per ml; velocity is in total cpm of $BaC^{14}O_3$ formed in the first 10 minutes; DPN, phosphate buffer pH 7.0, GSH are as in table I; and 7.4 mg protein as washed particle suspension. The final volume was 2 ml.

INHIBITOR STUDIES: Pea formic dehydrogenase has been found to be very susceptible to CN^- and azide (4). These inhibitors are rather unusual for a dehydrogenase. Cabbage particles were pre-incubated with various inhibitors at pH 7.0 for 10 minutes and then added to the formate reaction mixture. Table II shows that KCN and Na azide were extremely effective inhibitors at 10^{-3} M final concentration. The oxidation of formate was almost completely inhibited. Semicarbazide and hydroxylamine produced significant inhibitions at 5×10^{-3} M. To a certain extent 8-hydroxy quinoline stimulated the activity. If the particles were not pre-incubated with the inhibitor, but added directly to the reaction mixture plus inhibitor, substantially the same results were obtained, except that inhibition by hydroxylamine was only 29 % and the 8-hydroxy quinoline stimulation declined to 19 %.

TABLE II

EFFECT OF VARIOUS INHIBITORS ON FORMATE OXIDATION

INHIBITOR AND FINAL CONCENTRATION	RELATIVE ACTIVITY
No inhibitor	100
1×10^{-3} M KCN	1
1×10^{-3} M Na azide	0.1
5×10^{-3} M semicarbazide	41
5×10^{-3} M hydroxylamine	58
1×10^{-3} M 8-hydroxy quinoline	125

The reaction mixture contained 100 μmoles phosphate pH 7.0; 1 μmole Na formate-C^{14} (62,000 total counts); 0.2 μmole DPN; 20 μmoles GSH; particle suspension containing 6.8 mg. protein; distilled water to final volume of 2 ml. The particles were incubated with the phosphate buffer and the indicated inhibitor for 10 minutes prior to addition to the reaction mixture. The particles used for the control reaction without any added inhibitor were incubated with buffer and distilled water to the same final volume as where inhibitor was used. The center well contained a KCN: KOH mixture described by Krebs (6) when KCN was added to the reaction mixture. Incubation time was 30 minutes.

OXIDATION UNDER AEROBIC VS. ANAEROBIC CONDITIONS: The necessity for oxygen as a requirement for the conversion of formate to CO_2 was examined. The reaction was carried out in double sidearm Warburg vessels. The enzyme suspension was placed in one sidearm and tipped in to start the reaction. One pair of vessels was gassed with nitrogen for 5 minutes. One of the pair contained methylene blue to act as an electron acceptor. Another pair of vessels of similar composition was not gassed with nitrogen, but kept under the same conditions of manipulation and equilibration. A third pair of vessels was used as a control in that the enzyme suspension was tipped in at once.

TABLE III

FORMATE OXIDATION UNDER ANAEROBIC CONDITIONS

REACTION SYSTEM	FLASK ATMOSPHERE	TOTAL COUNTS/MG PROTEIN
Complete, enzyme tipped in immediately	Air	706
Same, plus methylene blue	"	665
Complete, enzyme tipped in after gassing 5 min with N_2 and 10 min equilibration	Nitrogen	216
Same, plus methylene blue	"	789
Complete, enzyme tipped in after 15 min in air in sidearm	Air	582
Same, plus methylene blue	"	610

Complete system contained 100 μmoles phosphate, pH 7.0; 1 μmole Na formate (62,000 total counts); 20 μmoles GSH; 0.2 μmole DPN; 0.5 ml washed particle suspension containing 9.6 mg protein; distilled water to a final volume of 2 ml. Where indicated, 0.2 ml of a 0.5 % methylene blue solution was added. After the enzyme was tipped in, the reaction, was carried on for 30 minutes at 30° C before stopping the incubation by tipping in the H_2SO_4 from the other sidearm.

This was to determine whether there was a large amount of inactivation of the enzyme during the time of the gassing and equilibration period. The results are summarized in table III. If the gas phase was air, then there was no effect (or only a slight one) on adding methylene blue. The oxidation of formate was reduced 60 to 70 % after gassing with nitrogen. Adding methylene blue raised the total oxidation back to the amount found under aerobic conditions.

TABLE IV

NUCLEOTIDE INHIBITION OF FORMATE OXIDATION

NUCLEOTIDE ADDED	RELATIVE ACTIVITY
None	100
ATP	77
ADP	62
AMP	47
Adenosine	84
Adenine	86
Yeast AMP	101
UTP	109

The reaction mixture was the same as that given in table III. 10 μmoles of the respective nucleotides were added as indicated. Incubation time, 30 minutes. 6.0 mg protein per vessel as a washed cytoplasmic particle suspension.

ADENINE NUCLEOTIDE INHIBITION: The inhibition of the particulate formate oxidation by ATP (table I) was examined in more detail. The effect of various nucleotides on the production of $C^{14}O_2$ is shown in table IV. The inhibition appears to be specific for both the presence of the adenine moiety and a phosphate on the 5′ position of the ribose. Uridine triphosphate (UTP) and yeast adenylic acid had no inhibitory effect. There was an increasing inhibition on going from ATP to adenosine-5′-phosphate (AMP). This may be a reflection of the ease of permeation by AMP compared to adenosine diphosphate (ADP) or ATP.

PEP carboxylase and PEP carboxykinase have been shown to be widely distributed in plant tissue (10). These enzymes catalyze reactions A and B, respectively.

A. PEP + CO_2 → oxalacetate + inorganic phosphate

B. PEP + ADP + CO_2 ⇄ oxalacetate + ATP

Plant PEP carboxykinase has a specific requirement for adenine nucleotides (10), whereas the avian and animal enzymes show a guanosine or inosine nucleotide specificity (2, 7). If reaction B above is the cause of the nucleotide inhibition, then added PEP should also give an inhibition. Table V clearly demonstrates a large inhibition on addition of PEP.

TABLE V

EFFECT OF PEP AND PGA ON PRODUCTION OF LABELED CO_2

COMPONENT ADDED	RELATIVE ACTIVITY
None	100
+ 10 μmoles ADP	62
+ 2 μmoles PEP	21
+ 2 μmoles PEP + 10 μmoles ADP	18
+ 2 μmoles PGA	36
+ 2 μmoles pyruvate	101

The reaction mixture was the same as described in table III. Incubation time was 30 minutes.

The production of $C^{14}O_2$ was inhibited 80 % in the presence of 10^{-3} M PEP. PGA was also inhibitory at the same concentration but to a lesser extent. The presence of enolase and phosphoglyceric acid mutase is indicated by this result. When pyruvate was added in the same concentration, there was no effect. If the inhibition is due to the fixation of the CO_2 produced, the action of PEP is not due to hydrolysis to pyruvate and fixation by means of malic enzyme. An attempt to demonstrate incorporation of label into the organic acid fraction in the presence of AMP or PEP was not successful. The fact that no activity could be demonstrated in the organic acid fraction might be a reflection of the rapid removal of the oxalacetate formed, such as by a rapid transamination to aspartate. Aspartate is labeled very rapidly by $C^{14}O_2$ in certain cases (15, 16).

SOLUBILIZATION OF FORMIC DEHYDROGENASE: The enzyme was solubilized from an acetone powder of the particles and by a digitonin extraction as described previously. Table VI compares the activity of the washed particles, the digitonin and acetone powder extracts of the particles, and the cytoplasm. The DPN requirement is readily apparent after solubilization. Triphosphopyridine nucleotide (TPN) cannot substitute for DPN. This specificity is the same as that found for pea formic dehydrogenase (9). The acetone powder extract showed a different effect on adding ATP than the digitonin extract. The stimulation of activity of the former was similar to the stimulation exhibited by the cytoplasmic system upon addition of ATP. The oxidation of formate by the digitonin extract was inhibited when ATP was present.

DISTRIBUTION OF PARTICULATE SYSTEM: A brief survey of several plant families was made to determine the presence and intracellular localization of formate oxidation. Pea seed was studied because it is the customary source of formic dehydrogenase. Special emphasis was placed on testing members of the Cruciferae (cabbage, turnip, radish, cauliflower) because of the negative results reported with seed extracts (3). Squash, representing the Cucurbitaceae, had also given negative results with seed extracts. Spinach leaf was included because it is commonly used as a source of chloroplasts and chloroplast fragments for enzymatic studies. In every case formate was oxidized and the activity almost exclusively localized in the particulate fraction (table VII). No attempt was made to determine and use the optimum conditions for oxidation for each preparation, so the specific activities are probably lower than could be obtained.

The pea seed dehydrogenase was the least concentrated in the particles, but this fraction still had three times the activity of the cytoplasm on a per milligram protein basis. The pea and cabbage cytoplasm fractions were prepared by a procedure which had been shown to stabilize the formic dehydrogenase of pea seed (4).

TABLE VI

COMPARISON OF SOLUBILIZED FORMIC DEHYDROGENASE WITH PARTICULATE AND CYTOPLASMIC SYSTEM

REACTION SYSTEM	TOTAL COUNTS/MG PROTEIN			
	WASHED PARTICLES	ACETONE POWDER EXTRACT	DIGITONIN EXTRACT	CYTOPLASM
Control	1,510	1,252	216	28
+ DPN	1,885	2,066	1,653	126
+ ATP	1,065	...	205	31
+ DPN + ATP	1,148	3,391	1,247	556
+ TPN	...	...	218	...
+ TPN + ATP	...	...	...	102

Control reaction mixture consisted of phosphate buffer, Na formate-C^{14}, GSH, and enzyme, as in table III. 0.2 μmole DPN or TPN and 10 μmoles ATP added where indicated. Incubation time was 60 minutes, except for the digitonin extract which was only 30 minutes.

DISCUSSION

The present work has shown that the widespread ability of plants to oxidize formic acid is not confined to the seeds. Extremely active preparations were made from other organs of plants which had no activity on the basis of seed extracts. The localization of formate oxidation in the particulates isolated by differential centrifugation from plant or animal[3] homogenates completes a unified biochemical pattern for localization of enzymes oxidizing this compound. Stickland (14) found that bacterial preparations also possessed formate-oxidizing ability which was localized in an insoluble cell fraction. Previous work on pea formic dehydrogenase was done under conditions which would not distinguish a particulate system from a soluble one. The procedure which had customarily been used was that of extracting a homogenate with an aqueous salt solution. Such procedures have frequently been used to solubilize enzymes from particulate structures (12). The results in table VII also indicate that pea seed particles are more fragile than others and the enzyme would tend to be solubilized more readily during homogenization.

Certain conclusions may be drawn concerning the electron transport system after formic dehydrogenase. Under anaerobic conditions methylene blue is as effective as oxygen as an electron acceptor. This indicates that a diaphorase must be present in the particles. Another implication of the results in table III is that endogenous electron acceptors must be constituents of the particles since under a nitrogen atmosphere the oxidation of formate was not lowered to a zero value.

The solubilized system showed a quite different ATP response depending upon the method of extraction. Acetone powder extracts behaved similarly to the cytoplasm in exhibiting a strong stimulation of formate oxidation when ATP was added. Digitonin extracts had the same response to added ATP as the washed particles. Cabbage particles have been shown to contain active pyrophosphatases (11). In the case of the acetone powder extracts the added ATP is probably protecting the DPN from destruction. The lack of effect of added ATP with digitonin extracts would imply that the latter treatment either inactivates or does not extract these enzymes.

[3] Mazelis, M. Unpublished experiments with mouse liver and kidney homogenates.

TABLE VII

INTRACELLULAR LOCALIZATION OF FORMATE OXIDATION IN ORGANS FROM SEVERAL HIGHER PLANTS

ENZYME SOURCE	TOTAL COUNTS/MG PROTEIN					
	WASHED CYTOPLASMIC PARTICLES			CYTOPLASM		
	No DPN	+DPN	+DPN +ATP	No DPN	+DPN	+DPN +ATP
Cabbage leaf	1,575	1,655	1,135	20	40	300
Pea seed	606	1,140	...	26	384	185
Spinach leaf	194	301	171	36	64	...
Radish root	673	1,229	921	26	47	...
Cauliflower bud	1,270	1,608	1,360	2	...	29
Turnip root	214	703	202	26	...	50
Squash fruit	1,284	2,715	1,605	180	206	18

Phosphate buffer, formate C^{14}, and GSH as in table III. 0.2 μmoles DPN and 10 μmoles ATP added where indicated. Cytoplasm was the lyophilised preparation described in "Methods" in the case of cabbage and pea seed. In all other cases an aliquot of the supernatant fluid obtained after removal of the particles from the homogenate by centrifugation at 23,000 × G was used as the source of enzyme. Incubated for 60 minutes at 30° C.

Many metabolic reactions lead to the formation of C_1 units which can be converted to formate (5). The widespread occurrence of PEP carboxylase and PEP carboxykinase in higher plants (10), and the particulate nature of enzymes fixing CO_2 in the presence of PEP (10, 18) has been well established. The presence of these enzyme complexes could be a means whereby the carbon in the formate or C_1 pool would be conserved.

Summary

Formate is oxidized by enzymes localized in the particles sedimenting at 23,000 × G from sucrose homogenates of organs of higher plants.

The enzyme has been found in various roots, buds, leaves, seeds, and fruits, and is DPN-specific.

The production of CO_2 from formate by cabbage particles is inhibited strongly in the presence of 10^{-3} M PEP or PGA, or 5×10^{-3} M adenine nucleotides, presumably by refixation of the CO_2 formed.

The enzyme is readily solubilized by a digitonin treatment of the particles.

Literature Cited

1. Adler, E., and M. Sreenivasaya. 1937. Uber die Komponenten der Dehydrasesysteme. XVI. Zur Kenntnis der Formicodehydrase und der Alkoholdehydrase in Planzensamen. Zeits. Physiol. Chem. 249: 24–39.
2. Bandurski, R. S., and F. Lipmann. 1956. Studies on an oxalacetic carboxylase from liver mitochondria. Jour. Biol. Chem. 219: 741–752.
3. Davison, D. C. 1949. Distribution of formic and alcoholic dehydrogenases in the higher plants—their variation in the pea plant during its life cycle. Proc. Linnean Soc. N. S. Wales 74: 26–36.
4. Davison, D. C. 1951. Studies on plant formic dehydrogenase. Biochem. Jour. 49: 520–526.
5. Fruton, J. S., and S. Simmonds. 1958. General Biochemistry, 2nd ed. P. 774. John Wiley, New York.
6. Krebs, H. A. 1935. Metabolism of amino acids. III. Deamination of amino acids. Biochem. Jour. 29: 1620–1644.
7. Kurahashi, K., R. J. Pennington, and M. F. Utter. 1957. Nucleotide specificity of oxalacetic carboxylase. Jour. Biol. Chem. 226: 1059–1075.
8. Lineweaver, H. and D. Burk. 1934. The determination of enzyme dissociation constants. Jour. Amer. Chem. Soc. 56: 658–666.
9. Mathews, M. B., and B. Vennesland. 1950. Enzymic oxidation of formic acid. Jour. Biol. Chem. 186: 667–682.
10. Mazelis, M. and B. Vennesland. 1957. Carbon dioxide fixation into oxalacetate in higher plants. Plant Physiol. 32: 591–600.
11. Mazelis, M. 1959. Enzymatic degradation of adenosine triphosphate to adenine by cabbage leaf preparations. Plant Physiol. 34: 153–158.
12. Morton, R. K. 1955. Methods of extraction of enzymes from animal tissues. In: Methods in Enzymology, S. P. Colowick and N. O. Kaplan, eds. Vol. 1. Pp. 25–51. Academic Press, New York.
13. Stumpf, P. K. 1955. Fat metabolism in higher plants. III. Enzymic oxidation of glycerol. Plant Physiol. 30: 55–58.
14. Stickland, L. H. 1929. The bacterial decomposition of formic acid. Biochem. Jour. 23: 1187–1198.
15. Suzuki, I. and C. H. Werkman. 1958. Chemoautotrophic carbon dioxide fixation by extracts of *Thiobacillus thiooxidans*. I. Formation of oxalacetic acid. Arch. Biochem. Biophys. 76: 103–111.
16. Warburg, O. 1958. Photosynthesis. Science 128: 68–73.
17. Weichselbaum, T. E. 1946. An accurate and rapid method for the determination of proteins in small amounts of blood serum and plasma. Amer. Jour. Clin. Pathol. (Tech. Sect.) 10: 40–49.
18. Young, L. C. T., and J. S. D. Graham. 1958. Carbon dioxide fixation in particulate preparations from barley roots. Nature 181: 1071.

EVALUATION OF LIGHT SOURCES FOR PLANT GROWTH [1, 2, 3]

A. T. LEISER, A. C. LEOPOLD, AND A. L. SHELLEY

DEPARTMENTS OF HORTICULTURE AND ELECTRICAL ENGINEERING, PURDUE UNIVERSITY, LAFAYETTE, INDIANA

Selection of light sources for installation in environmental control chambers for plants has generally been by empirical means. Early workers grew plants under various types of lamps and then selected the lamps providing the most normal looking plants (1). Later experiments established that combinations of fluorescent and tungsten lamps resulted in superior plant performance, and these lamp combinations have been installed in many growth chambers (8). More recently, comparisons of various spectral regions at uniform illumination intensities have been made to provide a more precise means of selection of light sources (6, 4).

Faced with the problem of selecting a light source for ourselves, we have developed a simple method of making a comparative evaluation, and have employed it for combinations of some currently available lamps. The basis of the method is to measure the growth rates of plants under a series of light intensities for each light source, and then by plotting a growth/light intensity curve for each source, to compare the light sources by the position and slope of the response curve for each.

The total radiant power (watts) in the visible spectrum has been used in reporting the growth responses. The simplest means of measuring light intensity or illumination, however, is by means of a foot-candle meter. While this method does not correct for differences in spectral distribution, it offers great convenience. Hence, the foot-candles produced with the several light sources are also given for easy comparison.

Some recently developed lamps were evaluated. Certain combinations of these lamps gave initial intensities as high as 5,000 ft-c at the plant level. High output fluorescent lamps (General Electric Power Groove F48PG17/CW) were used alone and in combination with tungsten and deluxe (color-improved) mercury vapor lamps (General Electric H400–RC1). As a tungsten source, 150 watt reflector floods were used alone, or 75 watt inside-frosted lamps were used in combinations with the other bulbs. The number and kind of lamps in each combination together with the three intensities obtained and total watts radiated for each are in table I. The intensities were measured with a Weston model 603 light meter at the end of each experiment. About 10 to 15 % reduction from initial intensities had occurred when a water filter was used. The approximate spectral distribution of the light provided by these various lamps and combinations is given in figure 1.

The experimental chamber was equipped with a refrigeration unit and an electric heater for temperature control. Thermostats held the air temperature at 78° ± 2° F. The light plenum was separated from the growing chamber by either a glass or a two mil polyethylene plastic baffle. In order to increase intensities the fluorescent lamps were staggered allowing overlapping of tubes. Additional lamps were also placed at right angles to the main axis on the sides of the lamp plenum to obtain more even illumination. Tungsten and mercury vapor lamps were hung between and below the fluorescent tubes.

The growing area (4 ft × 4 ft) was divided into three blocks, each receiving a different intensity of light. The reductions from full light were obtained with two neutral density screens (Saran—"Lumite" No. 1804-002 and No. 1808-002) rated as producing 51 % and 72 % actual shade respectively. Differences in orientation of emission resulted in considerable variation from these ratings (table I).

A monocotyledenous plant (Knox wheat) and a dicotyledenous plant (red kidney bean) were used as indicator plants. These species were selected on the evidence that they would show minimum morphological aberrations when exposed to various types of light (3).

Seeds were sown in plastic pots in a standardized mix of peat moss and sand and fertilized with high analysis soluble fertilizer. Germination and early growth were in a greenhouse at 60° F night temperature. The experiments were started when the third leaf of the wheat was developing and the first internode above the seed leaves of the bean was just starting expansion. This was about 13 ± 3 days after sowing. Each pot contained either three wheat plants or one bean plant. The plants of each species were divided into five groups of 12 pots each. One group was used to determine initial dry weights, one group remained in the greenhouse (control), and one group

[1] Received revised manuscript August 31, 1959.

[2] Journal Paper No. 1417, Purdue University, Agricultural Experiment Station, Lafayette, Ind.

[3] This reasearch was supported in part by a grant from the Purdue Research Foundation. Contributions of equipment by the General Electric Co., Large Lamp Division, Nela Park, Cleveland, Ohio, and Ballast Division, Danville, Ill., are gratefully acknowledged.

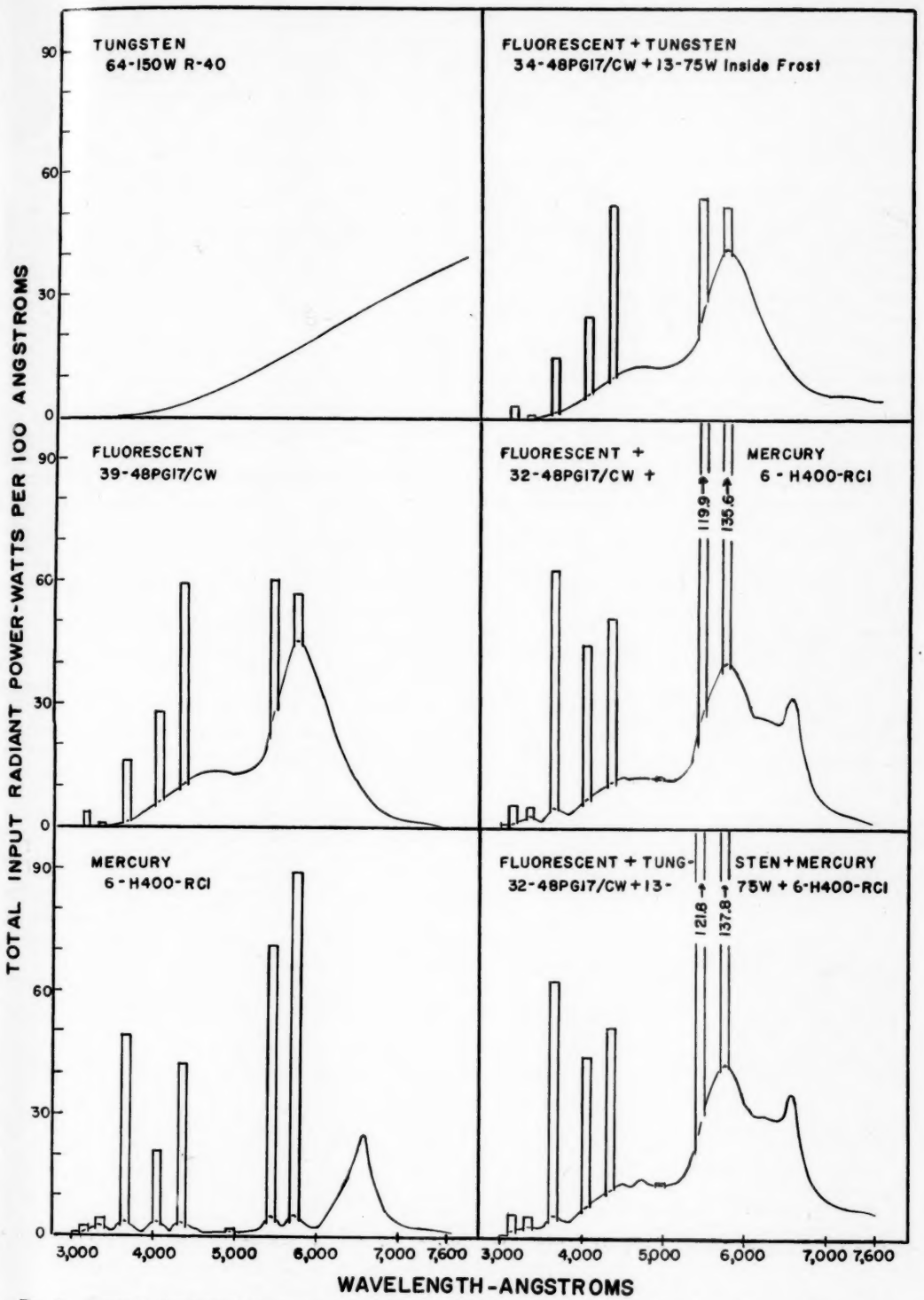


FIG. 1. Spectral distribution of several lamps and combinations used in growth chamber. These data are calculated from the manufacturer's (General Electric Co.) spectral distribution curves.

was transferred to each light intensity in the growth chamber.

The experiments were conducted with a day length of 14 hours. Extension of day length in the greenhouse was by tungsten light. At the end of 10 days the plants were measured, harvested, and fresh and dry weights determined.

The growth rates of wheat grown under four different light sources are plotted in figure 2. Each light source is represented as a separately plotted line. The growth rates obtained with wheat under the fluorescent bulbs alone are much smaller than those under tungsten or the several combinations of lamps tested. This illustrates the well known fact that improvement of growth results when fluorescent bulbs are supplemented with longer wave-length light. It is also very evident that light must be provided in a growth chamber at intensities far above those at which photosynthesis has been reported to be saturated (2, 8, 5, 7) or at which growth has been declared to reach a maximum (1) in order to obtain maximum growth rates. This is no doubt due in part to the random orientation of leaves and to shading.

Each increase in intensity under every light regime resulted in a highly significant increase in growth rate. The greatest growth rates of wheat were obtained under the highest intensities of light supplied. Maximum growth rate was under a light regime of Power Groove fluorescent plus deluxe mercury vapor lamps. Lack of any plateaus indicates that sufficient radiation input for maximum growth has not yet been obtained.

Growth rates for bean plants are presented in figure 3. Again the lesser effectiveness of the fluorescent bulbs alone is evident from the lower line and the lesser slope of the growth response curve. The bean plants were less responsive to high intensities than were the wheat plants, no doubt in part due to

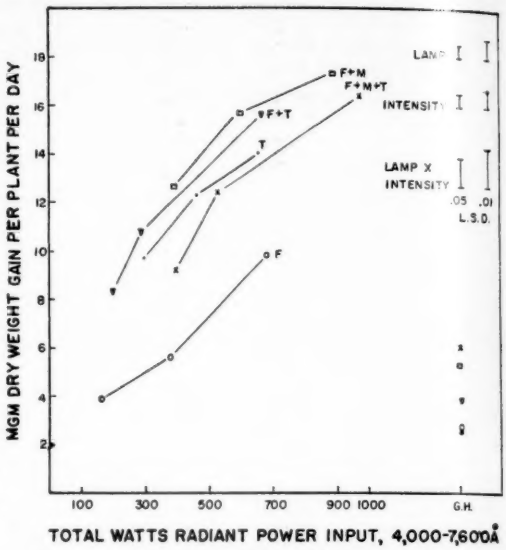


FIG. 2. Growth rate of Knox wheat grown under four light regimes for 10 day periods. Dry weights gain (as mgm/plant/day) plotted against the total radiant power input in the visible spectrum. Control plants grown in greenhouse (GH) are compared with tungsten (T), fluorescent (F), fluorescent plus tungsten (F + T), fluorescent plus deluxe mercury vapor lamps (F + M) and fluorescent, tungsten and deluxe mercury vapor lamps (F + M + T).

TABLE I

EXPERIMENTAL LIGHT SOURCES USED, INTENSITIES OBTAINED (FT-C.), AND RADIANT POWER (WATTS PER 16 SQ FT) INPUT IN VISIBLE SPECTRUM (4,000-7,600 Å)

LAMPS	FILTER	INTENSITIES (FT.-C.)			RADIANT POWER (WATTS)*		
		FULL	MEDIUM	LOW	FULL	MEDIUM	LOW
Tungsten-64—150 W. reflector floods	Water	2,400	1,700	1,100	654	463	298
Fluorescent-39—G. E. No. 48PG17/CW	Polyethylene	4,200	2,200	1,000	678	377	161
Fluorescent + tungsten 34—G.E. 48PG17/CW + 13—75 W. inside frost	Water	3,600	1,600	1,100	660	293	202
Fluorescent + mercury 32—G.E. 48PG17/CW + 6—G.E. H400–RC1	Water	4,500	3,000	2,000	889	596	397
Fluorescent + tungsten + mercury 32—G.E. 48PG17/CW + 13—75W + 6—G.E. H400–RC1	Water	4,400	2,400	1,800	974	531	398

* Calculated from manufacturer's (General Electric Co.) spectral distribution curves.

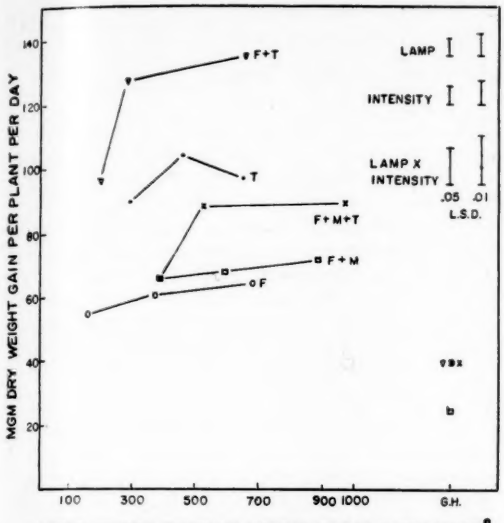


FIG. 3. Growth rate of red kidney bean grown under five light regimes for 10 day periods. Dry weight gain (as mgm/plant/day) plotted against total radiant power input in the visible spectrum. Control plants grown in greenhouse (GH) are compared with five lamp combinations as in figure 2.

the horizontal positioning of the bean leaves in contrast to the more random orientation of the wheat leaves. Some injury to bean leaves occurred in the highest intensities. This appeared to be due in part to radiant heat. Preliminary evidence indicates that much improvement of bean responses to high light intensities can be obtained with a more generous supply of nutrients and hardening of the plants.

The growth curves indicate the beneficial effect of high light intensities in environmental growth chambers. Growth at the usual levels of light in growth chambers (1,200-1,500 ft-c) was weak and spindly in comparison with growth in intensities above 4,000 ft-c. It is also interesting to note that while the bean plant does not respond to increases in fluorescent light alone above 1,000 ft-c, the addition of some supplemental lamps can greatly increase both the growth rate and the total radiant energy utilizable. Very high intensities have been obtained with Tungsten lamps alone (3) but the cooling problems involved limit the value of such an installation.

The evaluation of light sources by means of growth curves obtained simultaneously at several intensities appears to have particular merit. This method offers a practical alternative to the nearly insurmountable problem of adjusting varied light sources to equal energy levels. By its response, the plant indicates the cumulative effects of the varied light regimes on the many processes involved in growth. By comparing these responses at several intensities for each light regime, a more reliable index for evaluation is obtained than is possible by single intensity comparisons commonly used in the past. It is hoped that the method described here will be useful in development of improved light sources for growing plants.

LITERATURE CITED

1. CROCKER, W. 1958. Growth of Plants, Reinhold Publ. Co., New York.
2. MYERS, J. and G. O. BURR. 1940. Studies on photosynthesis. Some effects of high light intensity on Chlorella. Jour. Gen. Physiol. 24: 45–67.
3. PLATT, R. B. 1957. Growth chamber with light of solar intensity. Science. 126: 845.
4. VINCE, D., M. G. CLARKE, H. R. RUFF, and R. H. STOUGHTON. 1956. Studies on the effects of light quality on the growth and development of plants. I. Apparatus for the growing of plants under controlled light and temperature conditions. Jour. Hort. Sci. 31: 8–15.
5. VINCE, D., and R. H. STOUGHTON. 1957. Artificial light in plant experimental work. In: Control of the Plant Environment, J. D. Hudson, ed. Pp 72–83. Academic Press, New York.
6. WASSINK, E. C. and J. A. J. STOLWIJK. 1952. Effects of light of narrow spectral regions on growth and development in plants. Proc. Konikl. Ned. Akad. Wetenschap. 55: 472–488.
7. WASSINK, E. C. 1957. The study of plant growth in controlled environments. In: Control of The Plant Environment, J. P. Hudson, ed. Pp. 36–57. Academic Press, New York.
8. WENT, F. W. 1957. The Experimental Control of Plant Growth. Chronica Botanica Co., Waltham, Mass.

FACTORS AFFECTING GROWTH OF TOBACCO CALLUS TISSUE AND ITS INCORPORATION OF TYROSINE [1, 2]

DONALD K. DOUGALL [3] AND KOICHI SHIMBAYASHI

DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF WISCONSIN, MADISON

The elaboration of specific compounds for specific types of differentiation has been suggested to explain the production of roots, shoots, and flowers by plants. The search for compounds directly or indirectly influencing morphogenesis has produced only a few well defined substances such as indoleacetic acid, the gibberellins, and the kinins. Experiments using these compounds in tissue cultures have revealed first that the effect of the compound is not only dependent on its concentration in the medium, but also is dependent on the concentrations of other compounds present (15, 19, 22), and second that these growth regulating compounds influence a wide variety of anatomical (19), physiological (15), and biochemical (6, 16, 17, 18) responses of the cultured tissue. One of the most interesting systems described (19) is that in which the production of buds or shoots by tobacco callus tissue in culture is influenced by the concentration of the four factors: indoleacetic acid, kinetin, phosphate, and tyrosine. The present paper reports an investigation of the growth of tobacco callus cultures and of the fate of C^{14}-labeled tyrosine in this system.

METHODS

TISSUE CULTURES: Callus tissue was obtained from Dr. F. Skoog. Cultures were maintained on the modified White's basal medium described by Skoog and Tsui (20) supplemented by increasing the KH_2PO_4 to 62.5 mg/l and by adding indoleacetic acid (2.0 mg/l) and kinetin (0.2 mg/l). The medium was adjusted to pH 5.4, then autoclaved at 120° C for 15 minutes. Cultures were maintained in a liquid medium in 20 × 150 mm Pyrex tubes. Each tube contained 15 ml of medium, and was closed by an aluminum cap. One piece of tissue in each tube was supported at the liquid surface (8) by a folded 90 mm circle of Whatman's No. 42 filter paper.

When solidified media were desired, 1 % agar was incorporated into the liquid media; 50 ml portions were used in 125 ml Erlenmeyer flasks. The flasks were closed with cotton plugs; three pieces of callus tissue were placed on the agar in each flask.

EFFECTS OF NUTRIENTS AND GROWTH FACTORS ON TISSUE: These experiments were a randomized complete block with ten replicates. Concentrations of tyrosine, indoleacetic acid, kinetin, and phosphate were varied. The change in fresh weight was followed by weighing each piece aseptically during the course of the experiment. Infection was judged only by visual inspections. The appearance of buds and shoots during the experiment also was recorded. At the termination, the total nitrogen in the tissue pieces of five of the ten replicates was determined; the tissue pieces were digested (9) and ammonia in aliquots of the final distillate was estimated by the method of Moore and Stein (14).

FRACTIONATION OF TISSUE: Tissue pieces were blotted dry, weighed, and homogenized in 3 to 10 volumes of chosen buffer with a TenBroeck homogenizer. The fraction designated "cell walls" was recovered either by filtration through a fritted disk Pyrex funnel (porosity M, Corning Glass Works) or by centrifuging at 1,000 × G for 10 minutes. After cell walls were washed two or three times with the homogenizing medium the washings were combined with the supernatant.

In experiments where cell walls were extracted with solvents, the cell wall residue was recovered and washed with the solvent in the manner described for the cell walls.

The fraction designated "soluble protein" was prepared from combined washings and supernatant from the cell walls preparation by adding three volumes of ethanol and heating the solution to boiling. After cooling, the denatured soluble protein was collected by centrifugation at 5 to 10,000 × G for 15 minutes; the pellet then was washed two or three times in the centrifuge tube with 75 % ethanol.

The combined alcoholic supernatant and washings were concentrated in a climbing film vacuum evaporator to give the fraction designated soluble amino acids. When desired, this fraction was desalted by the method of Buchanan (7) using columns containing 5 ml of settled Dowex 50 X4 (H^+ form).

HYDROLYSIS OF FRACTIONS: To liberate the amino acids, fractions were hydrolyzed in redistilled constant boiling hydrochloric acid (approximately 1 ml/g fresh tissue) in sealed tubes at 120° C for 6 hours. Excess acid was removed by evaporating in a vacuum desiccator over flake NaOH; the humin was removed from samples for quantitative analysis by filtering aqueous extracts of the hydrolysate through fritted disk funnels (porosity F, Corning Glass Works). Filtrate and washings were combined and evaporated to dryness.

Samples of the soluble amino acids for quantitative analysis were hydrolyzed at 120° C for 15 minutes in 2 *N* hydrochloric acid to convert glutamine and as-

[1] Received September 1, 1959.

[2] Published with approval of the director of the Wisconsin Agricultural Experiment Station. Supported in part by research grant E–848 from the Division of Research Grants, National Institutes of Health, Public Health Service.

[3] Present address: Department of Biochemistry, University of Sydney, Sydney, Australia.

paragine quantitatively into ammonia and the corresponding dicarboxylic amino acids.

PAPER CHROMATOGRAPHY: Samples on Whatman's No. 1 chromatography paper were subjected to descending chromatography using *n*-butanol-acetic acid-water (4:1:5 v/v) (24) in the first dimension and followed by phenol-buffer (5:1 w/v; the aqueous buffer consisted of 6.3% sodium citrate, 3.7% KH_2PO_4, and 0.5% ascorbic acid) (3). The amino acids were revealed by spraying the air dried papers with a solution of 0.5 g ninhydrin in 250 ml *n*-butanol and 12.5 ml 2 *N* acetic acid when the phenol solvent was used, and with 0.16 g ninhydrin in a solution of 0.16 ml collidine in 100 ml *n*-butanol saturated with water if only the butanol-acetic acid solvent was used.

The hydrolysates were co-chromatographed with authentic samples of amino acids to establish the identity of the compounds revealed.

QUANTITATIVE ANALYSIS OF AMINO ACIDS: The method of Moore, Spackman, and Stein (13) was used with minor modification. Dowex 50 X8 (−400 mesh) was used in the 150 cm column and gave satisfactory resolution of tyrosine and phenylalanine only when operated at 50° C with the pH 3.18 buffer until 320 ml of effluent had been collected, and then at 60° C and pH 4.25 until a further 360 ml of effluent had been collected. The 15 cm columns were operated as described (13) for the estimation of basic amino acids in protein hydrolysates and the 50 cm column as described by Spackman, Stein, and Moore (21) for the chromatography of the soluble amino acids. The effluent was collected in 2.0 ml fractions; 1.0 ml samples were analyzed for amino acids by the method of Moore and Stein (14). The combined color yield and recovery from the columns for each amino acid was estimated using the standard mixture of amino acids prepared by Spinco Division, Beckman Instruments Inc., supplemented by samples of other amino acids (California Corporation for Biochemical Research) where necessary.

Hydroxyproline, which does not resolve completely from aspartic acid when chromatographed as described above, was estimated on the combined fractions from the aspartic acid region by re-chromatography at 30° instead of 50° C; the resolution is virtually complete at 30°. Hydroxyproline gives a yellow color with ninhydrin and was estimated by measuring optical densities at 440 mμ, whereas the optical density of the blue color from aspartic acid was measured at 570 mμ. Corrections in color yield were made for any small areas of overlap between peaks.

After chromatography of aliquots of the soluble amino acids on 150 cm columns, the remainders of the fractions from each peak were combined and desalted (7). Paper chromatography with authentic specimens validated the peaks assigned the identification of aspartic acid, threonine, serine, glutamic acid, glycine, alanine, and tyrosine.

DETERMINATION OF RADIOACTIVITY: All samples counted were prepared in the 0.2 *N* buffers used to elute the 150 cm columns. Aliquots of these solutions were mixed with an equal volume of 2% gelatin in 0.3 *N* (NaOH) and 1.0 ml of the resulting solution was dried on a copper planchet 1.0 inch in diameter. All samples were counted with the same counter for a minimum period of five minutes, and the results were expressed as counts/minute/ml corrected for coincidence loss where necessary and for background.

The specific activity of the amino acids was obtained by plotting the concentration of amino acid in each of the effluent fractions against the radioactivity in counts/minute/ml for the corresponding fraction. The slope, and the standard deviation, of the best line through the resulting points was calculated by the method of least squares assuming that concentration is the more accurate estimate. The slope of the best line represents the specific activity of the amino acid and the units used herein are counts/minute (corrected)/μmole of amino acid.

PREPARATION OF TYROSINE-C^{14} (BIOSYNTHESIZED): Chlorella pyrenoidosa was grown in a medium (McBride, L. private communication, 1958) containing/1: NH_4NO_3, 2.0 g; $MgSO_4 \cdot 7\ H_2O$, 0.308 g; KH_2PO_4, 1.96 g; K_2HPO_4, 3.43 g; $CaCl_2 \cdot 2\ H_2O$, 0.073 g; $KHCO_3$, 1.25 g. Iron was provided in the ferric form at 5 ppm and chelated by EDTA; the micro elements were provided by 0.5 ml/l of Hoagland's Solution A5 (Higher plant strength diluted 1/25).

The phosphates were adjusted to pH 6.2, and the remaining nutrients without $KHCO_3$ were autoclaved separately at 120° C for 30 minutes. The $KHCO_3$ was saturated with CO_2, filtered through a Morton bacterial filter and added aseptically to the medium.

To biosynthesize tyrosine, the algae were grown in four liters of medium containing 2.5 mc C^{14}/1 as $KHCO_3$. Growth was allowed to continue until complete, and the algae were collected by centrifugation, and hydrolyzed with acid. The hydrolysate was chromatographed on Dowex 1 X10 (50 × 2 cm column) (2, 10) and eluted with 0.1 *N* acetic acid. Fractions from 82 to 112 ml of effluent contained tyrosine and yielded 9.3 mg of radiochemically pure tyrosine, as shown by paper chromatography. Tyrosine was chromatographed on Dowex 50 X8 (150 × 1 cm); its specific activity was 106.5 ± 2.8 × 10^3 cpm/μM.

INCORPORATION OF TYROSINE-C^{14} INTO CULTURED TISSUE: The medium used in all the experiments with radioactive tyrosine had the same composition as that in which the tissue had been maintained except for the addition of tyrosine at 100 mg/l.

When uptake of tyrosine by cultured tissue was to be followed, slices of callus were cut and put into a sterile medium containing DL-tyrosine-2-C^{14}. The suspensions were shaken at room temperature, sampled aseptically at intervals, and the residual radioactivity was measured under the standardized conditions.

Biosynthesized tyrosine was used when the distribution of radioactivity among fractions of the tissue

was studied. For this experiment two 125 ml respirometer vessels with alkali wells were used. Each vessel contained 25 ml of medium, about 8 g of tissue, and tyrosine yielding 2.2×10^6 cpm; they were shaken for 24 hours at a temperature of 24 to 26° C. The respired carbon dioxide was trapped in 0.5 ml of 2 *N* sodium hydroxide in the well. The sodium hydroxide recovered at the end of the experiment was made to a standard volume, and counted.

After shaking for 24 hours, the medium from each flask was sampled aseptically. It then was diluted 1/10 with medium and 0.5 ml plated on tryptone-agar medium and incubated. No colonies appeared on any of the plates. The tissue recovered by filtration was fractionated. Radioactivity of each of the fractions was determined.

Results

Growth of Cultured Tissues: Two experiments employed various concentrations of phosphate, indoleacetic acid, kinetin, and tyrosine in the modified White's basal medium. Concentrations (mg/l) and times of weighing tissues were:

	Exp. XV	Exp. XVIII
KH_2PO_4	62.5, 250.0	250.0
Indoleacetic acid	1.0, 2.0	1.0, 2.0, 4.0
Kinetin	0.2, 0.4, 1.0	0.2, 1.0
Tyrosine	0, 200.0	0, 25.0, 100.0
Times of weighing (weeks)	0, 2, 4, 8	0, 2, 6

Summarized results for experiment XV are given in table I and for experiment XVIII are given in table II. Table III summarizes the two experiments.

Table I

Experiment XV. Growth of Tobacco Callus Tissue in Culture*

		$T_{0.0}$		$T_{200.0}$	
		$P_{62.5}$	$P_{250.0}$	$P_{62.5}$	$P_{250.0}$
$K_{0.2}$	$I_{1.0}$	2.38	2.83	1.96	1.80
	$I_{2.0}$	2.64	2.47	1.91	2.00
$K_{0.4}$	$I_{1.0}$	2.18	2.30	1.93	1.65
	$I_{2.0}$	1.85	2.14	1.98	1.95
$K_{1.0}$	$I_{1.0}$	1.87	1.91	1.79	1.80
	$I_{2.0}$	1.64	1.86	1.65	1.90

T = tyrosine; P = KH_2PO_4; subscript = mg/l; K = kinetin; I = indoleacetic acid.

Coefficient of variation 18 %.

*Ratio of final to initial weight in each treatment after 2 weeks of growth. (Average of 10 replicates).

Treatments giving differences significant at 1 % were kinetin, tyrosine, and kinetin-tyrosine interaction; and at 5 % was the indoleacetic acid-tyrosine interaction.

At 4 weeks, tyrosine-phosphate and indoleacetic acid-tyrosine-phosphate interactions were significant at 5 %, and after 8 weeks of culture the former had become significant at 1 %.

Table II

Experiment XVIII. Growth of Tobacco Callus Tissue in Culture*

		$T_{0.0}$	$T_{25.0}$	$T_{100.0}$
$I_{1.0}$	$K_{0.2}$	2.08	2.26	2.21
	$K_{1.0}$	1.96	2.16	2.31
$I_{2.0}$	$K_{0.2}$	1.94	2.24	2.34
	$K_{1.0}$	1.67	2.00	2.17
$I_{4.0}$	$K_{0.2}$	1.94	1.85	2.12
	$K_{1.0}$	1.72	1.78	2.13

Symbols as in table I. Coefficient of variation 14 %.

* Ratio of final to initial weight in each treatment after 2 weeks growth. (Average of 10 replicates).

Treatments giving differences significant at 1 % were indoleacetic acid, tyrosine, and kinetin; and none were significant at 5 %.

The effect of indoleacetic acid could only be demonstrated between 2.0 mg/l and 4.0 mg/l (significant at 1 %). After 6 weeks growth, the interactions kinetin-tyrosine and kinetin-tyrosine-indoleacetic acid gave differences significant at 5 %.

Table IV summarizes the results of total nitrogen determinations on the tissue pieces from experiment XV after 11 weeks of culture.

Detailed analysis of variance showed that the absolute growth of the tissue in experiment XV was different from that in experiment XVIII. Analysis also revealed, however, (table III) that there was no significant difference due to an interaction between the variable experiment and the levels of indoleacetic acid or the levels of kinetin used. This shows that, although the growth of the tissue differed in the two experiments, the relative effects of the two levels of each growth substance were the same in each case. Therefore, the results of the two experiments can be combined to give the following conclusions:

I. Kinetin at concentrations above 0.2 mg/l suppresses the fresh weight gain of the tissue.

II. Indoleacetic acid gives the maximum increase in fresh weight at concentrations between 1.0 and 2.0 mg/l, whereas 4.0 mg/l is definitely inhibitory.

III. At concentrations up to 100 mg/l, tyrosine increases the fresh weight gain of the tissue, but at 200 mg/l the amino acid inhibits the weight increase.

IV. Whether 62.5 or 250 mg/l of KH_2PO_4 is supplied has little influence on the fresh weight of the tissue.

Although variation in levels of the growth hormones and tyrosine produced marked effects on the growth of the tissue in terms of fresh weight and total nitrogen, the number of tissue pieces in any one treatment which produced buds or shoots never exceeded two.

The production of buds or shoots by cultured tissue was compared in the presence and absence of 1 % agar. White's basal medium was supplemented with 250 mg/l KH_2PO_4 and 1.0 mg/l indoleacetic acid. Combinations of two levels of kinetin (0.2 and 1.0

TABLE III

COMPARISON OF GROWTH OF TOBACCO CALLUS TISSUE IN TWO EXPERIMENTS*

		$I_{1.0}$	$I_{2.0}$
Experiment XV	$K_{0.2}$	2.83	2.47
	$K_{1.0}$	1.91	1.86
Experiment XVIII	$K_{0.2}$	2.08	1.94
	$K_{1.0}$	1.96	1.67

*Ratio of final to initial weight in each treatment after 2 weeks growth. (Average of 10 replicates).

Symbols as in table I. Medium—White's basal medium + KH_2PO_4 250 mg/l and no tyrosine.

Treatments giving differences significant at 1 % was kinetin; and at 5 % were indoleacetic acid and "experiment".

There were no significant differences in the interactions of the three variables.

mg/l) and two levels of tyrosine (0.0 and 200.0 mg/l) were used; cultures were maintained in darkness. After 63 days, no tissue grown in the absence of tyrosine had produced buds or shoots. Tissue grown in liquid medium containing tyrosine had not produced buds or shoots. Tissue pieces cultured on 1 % agar however, had produced buds or shoots. On 0.2 mg/l kinetin and 200 mg/l tyrosine, 1/15 of the tissue pieces produced buds or shoots in 45 days and 5/15 in 63 days, whereas with 1.0 mg/l kinetin and 200 mg/l tyrosine 11/15 of the tissue pieces had produced buds or shoots in 45 days and 15/15 in 63 days.

FRACTIONATION OF TISSUE: Fractionation of callus tissue and determination of the nitrogen in each fraction gave the following distribution: cell walls, 27 to 42 %; soluble protein, 11 to 21 %; soluble amino acids, 44 to 56 % of the total nitrogen. Amount of nitrogen in the cell wall fraction was influenced little with homogenization in the following media: water, 50 % ethanol, sodium borate (1.1 g/l) at pH 9.2 (12), M/15 phosphate at pH 6.00, 7.00, or 7.95, or 20 % glucose at pH 7.0. Likewise, the cell-wall nitrogen was affected little with any of the above solvents or with M/15 phosphate at pH 7.0 containing 10^{-3} M EDTA, 90 % phenol, 6 M urea, formamide, 0.1 M EDTA, 0.1 *N* sodium hydroxide, or 0.1 *N* hydrochloric acid at room temperature, or formamide at 100° C for 1 hour. When the cell walls were heated, however, at 100° C for 1 hour with 0.1 *N* NaOH or 90 % formic acid, then part of the wall material dissolved and the amount of nitrogen bound to the treated cell walls decreased. When the time of heating was doubled, there was no marked decrease in the amount of nitrogen remaining bound to the cell walls.

The amount of material extracted from the cell walls with 90 % formic acid at 100° C for an hour decreased markedly with successive treatments, as demonstrated by hydrolysis of the extracts and separation of the liberated amino acids by paper chromatography. The removal of nitrogenous material from the cell wall by formic acid was not due to liberation of free amino acids.

The nitrogen content and yield of residue after extraction of cell walls with 0.1 *N* NaOH or 90 % formic acid at 100° C for 1 hour suggested that the formic acid had extracted more non-nitrogenous material from the cell walls than had NaOH. This extraction of non-nitrogenous material could not be reduced by the ethanol precipitation procedure of Albanese (1).

When formic acid extracted cell walls which contained 18 mg N/g, were extracted with 0.1 *N* NaOH at 100° C for 1 hour, the nitrogen content decreased to 14.5 mg N/g dry weight. This residue contained hydroxyproline, as did a precipitate obtained by adjusting the NaOH extract to pH 4.5 with acetic acid, adding three volumes of ethanol and boiling. The supernatant after removal of this precipitate contained 43 % of the total nitrogen in the formic acid extracted cell walls used. This may be explained by hydrolysis of protein, but the small decrease in the nitrogen content of the formic acid extracted cell walls by NaOH extraction requires that a non-specific solubilization of the cell walls has also taken place. In most of the subsequent experiments, formic acid was used to extract cell walls. These extractions gave four fractions from the tissue; soluble amino acids, soluble protein, formic acid soluble cell wall material and cell wall residue.

The presence of amino acids in hydrolysates of the cell walls, formic acid soluble cell wall material and the cell wall residue was demonstrated by paper chromatography and the results are given in table V. Hydrolysates of the cell walls and the cell wall residue gave spots which co-chromatographed with hydroxyproline, whereas this spot was absent from chromatograms of the hydrolyzed formic acid soluble cell wall material.

Cell walls were extracted at 100° C for 1 hour with 0.1 *N* sodium hydroxide and then washed free

TABLE IV

TOTAL NITROGEN IN TOBACCO CALLUS TISSUE CULTURES GROWN FOR ELEVEN WEEKS*

		T_0		T_{100}	
		$P_{62.5}$	P_{250}	$P_{62.5}$	P_{250}
$K_{0.2}$	$I_{1.0}$	2.38	3.19	3.48	2.98
	$I_{2.0}$	3.36	3.48	3.52	4.36
$K_{0.4}$	$I_{1.0}$	2.67	3.49	3.17	1.92
	$I_{2.0}$	2.43	2.70	3.64	3.27
$K_{1.0}$	$I_{1.0}$	1.80	2.59	2.41	2.55
	$I_{2.0}$	1.81	2.01	3.40	2.78

* Average of five replicates; values corrected to an initial weight of 100 mg. Data as mg N. Tissues were those used in experiment XV, table I.

Symbols as in table I. Coefficient of variation 36 %.

Treatments giving differences significant at 1 % was kinetin; and at 5 % were tyrosine and indoleacetic acid-tyrosine interaction.

TABLE V

AMINO ACIDS OBSERVED ON PAPER CHROMATOGRAMS OF SOLUBLE AMINO ACID EXTRACT AND HYDROLYSATES FROM VARIOUS FRACTIONS OF CALLUS CELLS

AMINO ACID	CELL WALLS	CELL WALL MATERIAL SOLUBLE IN FORMIC ACID	CELL WALL RESIDUE	SOLUBLE PROTEINS	SOLUBLE AMINO ACIDS*
Alanine	+	+	+	+	+
β-Alanine					+
γ-Aminobutyric ac.					+
Arginine	+	+	+	+	+
Aspartic ac.	+	+	+	+	+
Asparagine					+
Glutamic ac.	+	+	+	+	+
Glutamine					+
Glycine	+	+	+	+	+
Hydroxyproline	+		+		
Lysine					+
Ornithine					+
Proline	+	+	+		
Serine	+	+	+	+	+
Threonine	+	+	+	+	+
Tyrosine	+	+	+	+	+**
Leucine + isoleucine + phenylalanine	+	+	+	+	
Lysine + histidine + ornithine	+	+	+	+	
Valine + methionine	+	+	+	+	

* Desalted by the method of Buchanan (7); unlike the other fractions, this material was not hydrolyzed.
** Observed when tyrosine was supplied in the medium.

of alkali with water. The residue was autoclaved in water at 120° C overnight and the nitrogen in the extract and the residue estimated. The extract contained 0.83 mg and the residue 6.4 mg nitrogen.

CELL WALLS OF TOBACCO PITH CELLS: Pith tissue was removed from stems of tobacco plants (var. Wisconsin No. 38) as described by Bryan and Newcomb (6). The cell walls from this material and from callus tissue cultured for 4 weeks were isolated in M/15 phosphate at pH 7.0 and washed six times with the medium. The nitrogen in the cell walls expressed as a percentage of the total nitrogen was 2.3 % for the pith and 30 % for the callus tissue. When the cell walls were isolated by washing three times with the phosphate buffer used for the homogenization and then three times with 0.1 *N* NaOH at room temperatures, the nitrogen content was 1.7 % for the pith and 21 % for the callus tissue.

INCORPORATION OF RADIOACTIVE TYROSINE INTO TISSUE: When the uptake of radioactive tyrosine

TABLE VI

DISTRIBUTION OF RADIOACTIVITY BETWEEN FRACTIONS OF TOBACCO TISSUE*

	EXPERIMENT 1		EXPERIMENT 2	
Tissue weight (mg)	8,120		740	
Initial radioactivity (total cpm × 10^{-4})	220		220	
Fraction	*cpm* × 10^{-4}	*% Initial*	*cpm* × 10^{-4}	*% Initial*
Respired carbon dioxide	8.2	3.7	7.6	3.5
External medium	9.0	4.1	10.7	4.9
Soluble amino acids	101	45	102	45
Soluble proteins (by difference)	10	4.5	15.5	7.0
Formic acid soluble cell wall material	23	10.5	23	10.4
Cell wall residue	38.0	17.3	19	8.7
Total	189	85	178	80

* Cultured for 24 hours in the presence of labeled tyrosine and a complete growth medium.

was followed, it was found that the radioactivity in the medium described a curve of decreasing slope, so that approximately two-thirds of the radioactivity had disappeared after 24 hours. Therefore, slices of tissue were kept in the tissue culture medium with biosynthesized tyrosine for 24 hours and then fractionated. The results of the measurements of radioactivity in the fractions from the duplicate experiments are shown in table VI.

The external medium and the soluble amino acids were desalted according to Buchanan (7); their C^{14} distribution is shown in part A of table VII. The soluble proteins, formic acid soluble cell wall material, and a portion of the cell wall residue were hydrolyzed in constant boiling hydrochloric acid for 6 hours at 120° C. The radioactivity and free amino acids were estimated after removal of the excess acid and of the humin; these results are shown in part B of table VII.

The radioactivity and quantity of each amino acid in the hydrolysates of the soluble amino acids, soluble proteins, formic acid soluble cell wall material, and the cell wall residue are shown in tables VIII and IX. The only identified amino acid which was radioactive was tyrosine, although in the soluble amino acid fraction a second small peak of radioactivity appeared between glycine and alanine. The elution curve of the radioactivity of this compound was not exactly coincident with the elution curve for the alanine and the identity of the compound was not pursued.

Discussion

In the experiments described here concerning the growth of the tobacco callus tissue in culture, the interaction of several growth factors in influencing the growth of the tissue was demonstrated. The growth of the tissue either in terms of fresh weight or total nitrogen was influenced not only by the concentration of kinetin and tyrosine in the medium, but also by the combined effects of tyrosine with indoleacetic acid, with kinetin and with phosphate. The results of these experiments differ importantly from those described by Skoog and Miller (19), in that the production of buds or shoots was negligible. It was shown that growth of the callus on a medium containing 1 % agar led to the extensive production of buds or shoots by all callus pieces, whereas tissue pieces supported in the meniscus of the same medium in the absence of agar produced very few buds or shoots. No reason was found for this difference, and our studies on the incorporation of tyrosine into tissues were made with liquid media.

The first step in investigating tyrosine metabolism was to measure the specific activity of all the amino acids both free and bound in the tissue after feeding C^{14}-labeled tyrosine. The known partial destruction of amino acids when proteins are hydrolyzed in the presence of carbohydrate and the possibility that tyrosine would act as a precursor of the lignin of the cell wall (4) led to attempts to separate the proteinaceous material of the cell from insoluble materials which contained no protein. A variety of solvents failed to yield a nitrogen free insoluble residue. The final fractionation procedure used gave, in addition to the soluble amino acids, three protein-containing fractions which differed in their solubility properties. These have been called soluble proteins, formic acid soluble cell wall material, and the cell wall residue.

After acid hydrolysis and separation by ion exchange chromatography (13, 21), the quantity and radioactivity of every amino acid in each of the hy-

Table VII

Distribution of C^{14} Among Cell Fractions and Hydrolysates of Fractions Recovered from Tobacco Callus Tissue Supplied C^{14}-Labeled Tyrosine

	Experiment 1			Experiment 2		
	cpm × 10^{-4}	Recovery of C^{14}	μM*	cpm × 10^{-4}	Recovery of C^{14}	μM*
A. C^{14} in fractions separated on Dowex 50 (H^+)						
External medium						
Material not retained	3.0	33**		3.1	29**	
Amino acids	5.2	58	1.3	5.6	52	1.2
Recovery	8.2	91		8.7	81	
Soluble amino acids						
Material not retained	3.6	3.6		3.6	3.6	
Material displaced prior to amino acids	11.6	11.5		10.8	10.6	
Amino acids after hydrolysis	67.9	67.2	70	64.0	62.7	54
Recovery	82.1	82.0		78.4	76.9	
B. C^{14} in hydrolysates from cell fractions						
Solube proteins	2.3	23***	49	2.8	17***	49
Formic acid soluble cell wall material	12.4	54	243	12.2	53	290
Cell wall residue†	7.2	19	71	6.7	35	59

* μm of α-amino nitrogen determined by ninhydron (leucine standard).
** Recovery in each fraction as percentage of total radioactivity added to column.
*** Recovery as percentage C^{14} in each fraction relative to the C^{14} of entire tissue.
† Corrected to total cell wall residue.

TABLE VIII

RADIOACTIVITY OF TYROSINE IN FRACTIONS FROM TISSUES SUPPLIED C^{14}-LABELED TYROSINE*

	EXPERIMENT 1			EXPERIMENT 2		
	CPM/μMOLE × 10^{-4}	CPM-TOTAL × 10^{-4}	%**	CPM/μMOLE × 10^{-4}	CPM-TOTAL × 10^{-4}	%**
Original tyrosine	106.5 ± 2.8	220		106.5 ± 2.8	220	
Soluble amino acid-tyrosine	91.0 ± 11.9	32.1	32	118 ± 3.8	46	45
peak near alanine		0.34	0.34		0.41	0.40
Soluble protein-tyrosine	11.3 ± 0.88	1.2	12	11.1 ± 0.73	0.86	5.4
Formic acid soluble cell wall material-tyrosine	9.64 ± 0.24	3.7	16	10.0 ± 0.82	4.5	22
Cell wall residue-tyrosine	2.00 ± 0.21	0.45	1.2	2.10 ± 0.23	0.35	1.8
Total		38			52	
Total (% original cpm)		17			24	

* Standard deviations calculated by the method of least squares.
** % = cpm in amino acid as a percentage of the cpm in the fraction from which it was isolated.

drolyzed tissue fractions was estimated. Two of the fractions, the soluble proteins and the formic acid soluble cell wall material, differed little from each other in their amino acid composition (table IX) and rate of incorporation of tyrosine (table VIII). The cell wall residue appeared to represent a distinct entity, for it differed markedly from the other two proteinaceous fractions not only in having a lower rate of incorporation of tyrosine, but also in its amino acid composition and exclusive possession of hydroxyproline. The localization of hydroxyproline in this fraction is of interest, as Steward, Thompson, and Pollard (23) concluded that high levels of hydroxyproline in plant tissues are associated with high levels of metabolic activity. The contrasting high levels of hydroxyproline and the low rate of incorporation of

TABLE IX

AMINO ACID COMPOSITION OF TISSUE AND ITS FRACTIONS AFTER 24 HOURS IN PRESENCE OF TYROSINE-C^{14} (BIOSYNTHESIZED)**

	TISSUE AMINO ACIDS		SOLUBLE AMINO ACIDS		SOLUBLE PROTEIN		FORMIC ACID SOL.		CELL WALL RESIDUE	
	FREE	BOUND	1	2	1	2	1	2	1	2
Hydroxy proline	...	1.97	...	...	...	...	...	...	18.1	26.8
Aspartic acid	0.63*	3.27	8.2*	7.4*	8.6	8.5	7.5	7.5	4.9	4.6
Threonine	0.13	2.10	1.7	1.5	5.0	5.1	4.2	4.3	5.4	5.2
Serine	0.27	2.84	3.4	3.4	6.1	6.0	5.5	5.4	9.2	8.8
Glutamic acid	2.45*	3.66	31.6*	28.9*	9.9	10.1	8.6	8.8	3.8	3.7
Proline	0.02	2.83	0.5	...	5.8	4.8	6.3	6.4	6.1	6.7
Glycine	0.19	3.09	2.2	2.5	8.3	7.9	7.0	7.1	4.8	4.2
Alanine	0.39	2.95	4.9	4.7	8.6	8.1	6.8	6.8	4.0	3.6
Valine	0.11	2.65	1.2	1.7	7.0	7.3	5.3	5.3	6.5	6.2
Methionine	0.05	0.23	...	1.4	1.2	...	0.7	0.4	0.4	0.5
iso-Leucine	0.18	1.62	1.8	2.6	4.4	4.7	3.6	3.8	2.0	2.2
Leucine	0.09	2.70	1.3	0.9	7.7	7.6	6.1	6.5	3.3	3.2
Tyrosine	0.47	0.95	5.1	6.5	2.2	2.6	1.8	1.9	2.9	2.4
Phenylalanine	0.03	1.25	0.5	0.4	3.9	3.4	3.0	2.9	1.4	1.1
β-Alanine	0.11	...	1.8	1.1	...	...	...	...	...	...
Lysine	0.09	3.14	1.1	1.1	5.7	5.4	6.4	5.9	9.1	9.9
Histidine	...	0.87	...	...	1.4	1.4	1.7	1.9	2.3	2.7
Ammonia	2.23*	8.39	26.5*	28.3*	10.8	13.8	22.2	21.1	13.9	6.7
Arginine	...	1.48	...	...	3.4	3.5	3.4	3.7	2.1	1.4
γ-Amino butyric acid	0.67	...	8.4	8.0	...	...	...	...	...	...
% Recovery of ninhydrin color on chromatography			96-100	102-106	90	78	86	81	78	70

* These values represent the sum of the free compound and that present as the amide.
** Free and bound amino acids are expressed in μm/g fresh weight (average of exp. 1 and 2). Amino acids of the fractions are expressed as moles % of the amino acids recovered.

tyrosine into the cell wall residue suggests that their generalization cannot be extended to the protein fractions of tissues.

The protein fractions which were obtained in these experiments have different metabolic activities as indicated by the rates of replacement of tyrosine. These rates suggest that the most active fraction is the soluble protein, followed by the formic acid soluble cell wall material, and that the least active is the cell wall residue. Together the last two fractions, when isolated as cell walls from callus tissue, contained approximately 25 % of the total nitrogen, whereas cell walls from tobacco pith, which is regarded as nonproliferating, contained only 2 % of the total nitrogen. This difference between rapidly growing and nonproliferating tissues suggests that the cell walls fraction is important under conditions of high metabolic activity. Its primary function is not necessarily in intermediary metabolism but may be either in a storage or a structural role, for although the soluble proteins had the highest rate of replacement, they accumulated only 0.019 μM of tyrosine, while the combined formic acid soluble cell wall material and cell wall residue accumulated 0.065 μM of radioactive tyrosine in 24 hours.

In the preceding discussion the fate of tyrosine has been considered only in terms of its incorporation into proteins. However, in the analysis of the cell wall residue, the recovery of radioactivity in the liberated tyrosine (table VIII) was only 1.2 to 1.8 % of the radioactivity of the whole fraction. The recovery of radioactivity was 98 to 98.5 % when an authentic sample of radioactive tyrosine was chromatographed. The small percentage of the radioactivity of the fraction which is present as intact tyrosine residues would be consistent with the incorporation of tyrosine into the lignin of the cell wall. The incorporation of tyrosine into lignin has been observed by Brown and Neish (4), and it also has been shown that *p*-hydroxyphenylpyruvic acid is a precursor of the tyrosine in the cell wall protein, and simultaneously of the lignin of the cell wall (5) We hope to be able to report later our examination of the lignin of the cell wall residue and the extent to which tyrosine is incorporated.

The localization of hydroxyproline in the cell wall residue is of interest, for its presence in a protein has been thought to be characteristic of the collagen group of structural proteins (11), although some members of the keratin group contain hydroxyproline (3). Evidence against a similarity of the cell wall residue protein to collagen is its low levels of glycine and alanine, the presence of aromatic amino acids in the protein, and the fact that the residue cannot be converted into a soluble gelatin either in alkali at 100° C or in water at 120° C (11).

The amino acid composition of the cell wall residue differed from that of the other fractions in having lower levels of the dicarboxylic amino acids, glycine, alanine, valine, *iso*-leucine, leucine, and arginine, and higher levels of serine and lysine. In addition to these differences the molar ratio of tyrosine to phenylalanine in the cell wall residue was greater than one, whereas it was less than one in the other protein-containing fractions.

When the amino acid compositions of the three fractions are considered as a whole, the only outstanding features are the absence of cystine and the low and variable amounts of methionine present.

The specific activity data show that 12.5 % of the tyrosine in the soluble protein was replaced in 24 hours. Corresponding figures for the replacement of tyrosine in the formic acid soluble cell wall material and the cell wall residue were 10.5 % and 2.2 %, respectively. The specific activity of the free tyrosine shows that the tyrosine originally present was mixed with approximately six times as much added radioactive tyrosine during the course of the experiment. This large uptake of tyrosine by the tissue is also indicated by the low level of radioactivity in the external medium after 24 hours, and by the fact that the concentration of tyrosine in the tissue (0.43-0.51 μM/g fresh wt) is very close to the original concentration (0.55 μM/ml) in the medium. The radioactive tyrosine was provided to the tissue at such a high level that the amino acid pool of the tissue was overloaded, and thus the quantitative results presented here represent a response to an abnormally high level of tyrosine in the tissue. Qualitatively, the lack of appearance of carbon 14 in any amino acid but tyrosine shows that tyrosine is not an intermediate but an end product in amino acid biosynthesis.

Summary

The effect of varied concentrations of indoleacetic acid, kinetin, phosphate, and tyrosine on the growth and differentiation of tobacco callus tissue cultured in a liquid medium has been studied. The growth rate was influenced not only by the concentration, but also by the relative levels of each substance.

It was observed that callus tissue produced buds and shoots when grown on a medium containing agar, and it produced only callus when supported at the surface of a liquid medium of the same composition.

After fractionation of the callus tissue, 25 to 40 % of the total cell nitrogen remained associated with the insoluble cell wall fraction. This fraction was separated further by extraction with formic acid into two fractions which differed in amino acid composition, rate of incorporation of tyrosine, and in the presence or absence of hydroxyproline. The significance of these results is discussed in terms of tissue growth.

The incorporation of C^{14}-labeled tyrosine into the tissue was readily demonstrated during a 24 hour period of incubation. In this time, 12.5 % of the tyrosine in the soluble protein fraction had been replaced, 10.5 % in the formic acid soluble cell wall material and 2.2 % in the cell wall residue. No other amino acid became radioactive after the tissue had been cultured in the presence of C^{14}-labeled tyrosine for 24 hours.

Acknowledgements

The authors wish to thank Professors R. H. Burris and F. Skoog for their suggestions and encouragement, and Professors D. Grant and J. Torrie for their advice on statistical analysis.

Literature Cited

1. Albanese, A. A. 1944. Amino acid analysis of some common vegetables. Method for carbohydrate-free extraction of nitrogen from fresh vegetables. Anal. Chem. 16: 609–611.
2. Bilinski, E. and W. B. McConnell. 1957. Studies on wheat plants using C^{14} compounds. III. The utilization of acetate for amino acid biosynthesis. Can. Jour. Biochem. Physiol. 35: 357–363.
3. Block, R. J. and K. W. Weiss. 1956. Amino Acid Handbook, Charles C. Thomas, Springfield.
4. Brown, S. A. and A. C. Neish. 1955. Studies of lignin biosynthesis using isotopic carbon. IV. Formation from aromatic monomers. Can. Jour. Biochem. Physiol. 33: 948–962.
5. Brown, S. A., D. Wright, and A. C. Neish. 1959. Studies of lignin biosynthesis using isotopic carbon. VII. The role of *p*-hydroxyphenylpyruvic acid. Can. Jour. Biochem. Physiol. 37: 25–34.
6. Bryan, W. H. and E. H. Newcomb. 1954. Stimulation of methyl pectinesterase activity of cultured tobacco pith by indoleacetic acid. Physiol. Plantarum 7: 290–297.
7. Buchanan, D. L. 1957. Desalting amino acid solutions by displacement with piperidine. Anal. Chem. 29: 1877–1878.
8. Heller, R. 1953. Researches on the mineral nutrition of vegetable tissues cultivated in vitro. Ann. sci. nat. Onzième Sèrie Botanique et Biologii Végétale. 14: 1–223.
9. Hiller, A., J. Plazin, and D. D. Van Slyke. 1948. A study of conditions for Kjeldahl determination of nitrogen in proteins. Description of methods with mercury as catalyst, and titrimetric and gasometric measurement of the ammonia formed. Jour. Biol. Chem. 176: 1401–1420.
10. Hirs, C. H. W., S. Moore, and W. H. Stein. 1954. The chromatography of amino acids on ion exchange resins. Use of volatile acids for elution. Jour. Chem. Soc. 76: 6063–6065.
11. Kendrew, J. C. 1954. Structure proteins I. In: The Proteins, H. Neurath and K. Bailey, eds. Vol. 2. Pp. 846–949. Academic Press Inc., New York.
12. Lugg, J. W. H. 1939. The representativeness of extracted samples and the efficiency of extraction of proteins from the fresh leaves of plants; and some partial analyses of the whole proteins of leaves. Biochem. Jour. 33: 110–122.
13. Moore, S., D. H. Spackman, and W. H. Stein. 1958. Chromatography of amino acids on sulfonated polystyrene resins. An improved system. Anal. Chem. 30: 1185–1190.
14. Moore, S. and W. H. Stein. 1954. A modified ninhydrin reagent for the photometric determination of amino acids and related compounds. Jour. Biol. Chem. 211: 907–913.
15. Patau, K., N. K. Das, and F. Skoog. 1957. Induction of DNA synthesis by kinetin and indoleacetic acid in excised tobacco pith tissue. Physiol. Plantarum 10: 949–966.
16. Reinhold, L., and R. G. Powell. 1958. The stimulating effect of indoleacetic acid on the uptake of amino acids by tissue of *Helianthus annuus*. Jour. Exp. Bot. 9: 82–96.
17. Reinhold, L. 1958. Release of ammonia by plant tissues treated with indoleacetic acid. Nature 182: 1022–1023.
18. Reinhold, L. and R. G. Powell. 1956. A stimulating action of indole-3-acetic acid on the uptake of amino acids by plant cells. Nature 177: 658–659.
19. Skoog, F. and C. O. Miller. 1957. Chemical regulation of growth and organ formation in plant tissues cultured in vitro. In: Symposia of the Society for Experimental Biology. No. XI., The biological action of growth substances. H. K. Porter ed. Pp. 118–131. University Press, Cambridge, England.
20. Skoog, F. and C. Tsui. 1948. Chemical control of growth and bud formation in tobacco stem segments and callus cultured in vitro. Amer. Jour. Bot. 35: 782–787.
21. Spackman, D. H., W. H. Stein, and S. Moore. 1958. Automatic recording apparatus for use in the chromatography of amino acids. Anal. Chem. 30: 1190–1206.
22. Steward, F. C. and E. M. Shantz. 1954. The growth of carrot tissue explants and its relation to the growth factors in coconut milk. II. The growth promoting properties of coconut milk for plant tissue cultures. Année Biologique 30: 399–410.
23. Steward, F. C., J. F. Thompson, and J. K. Pollard. 1958. Contrasts in the nitrogenous compositions of rapidly growing and non-growing plant tissues. Jour. Exp. Bot. 9: 1–10.
24. Wotwood, A. J. 1949. A technique for examining large numbers of bacterial culture filtrates by partition chromatography. Jour. Gen. Microbiol. 3: 312–318.

INFLUENCE OF 2,4-DICHLOROPHENOXYACETIC ACID ON PATHWAYS OF GLUCOSE UTILIZATION IN BEAN STEM TISSUES[1, 2, 3]

S. C. FANG, FUAD TEENY, AND JOSEPH S. BUTTS

DEPARTMENT OF AGRICULTURAL CHEMISTRY, OREGON STATE COLLEGE

2,4-Dichlorophenoxyacetic acid (2,4-D) is the best known selective herbicide for effective control of broadleafed weeds in monocotyledonous crops. Although the mechanisms by which 2,4-D acts on plants have not been elucidated, several lines of investigation have shown: A. Stimulatory and inhibitory effects on the respiration of pea and oat tissues, depending upon the concentrations employed (6). B. Reduction of carbohydrate content of bean stem (7). C. Increased participation of pentose cycle pathway of glucose catabolism in root tips of pea, corn, and oat seedlings (4). These results suggested that carbohydrate metabolism may be greatly effected.

The present work is based upon the hypothesis that 2,4-D influences growth by affecting glucose utilization which furnishes the energy and/or building units for the synthesis of cellular constituents. This is approached by directly measuring utilization of radioactive carbon atoms from glucose in treated and untreated tissues after substantial morphological response has occurred. This paper reports the results from several experiments on the effect of 2,4-D on the pathways of glucose utilization in bean stem tissue using C^{14} labeled glucose.

MATERIALS AND METHODS

Bean plants (*Phaseolus vulgaris* var. Black Valentine) were grown in the greenhouse in metal flats containing Newberg sandy loam. The plants were treated when primary leaves were almost fully expanded and terminal buds were still very small. A 95 % ethyl alcohol solution containing 0.1 % of 2,4-D acid and 0.2 % polyoxethylene sorbitan monolaurate (Tween-20) was used for treatment. The solution was applied quantitatively by means of a microsyringe to each plant along the midrib of one primary leaf. Seven days after treatment, plants were harvested and first internodes were cut into 2 cm sections. Generally, two sections were cut from each plant. Approximately 20 sections, picked at random, were weighed and incubated immediately in 10 ml 0.01 M phosphate buffer (pH 5.2) containing 1 mg of radioactive glucose. Apparatus used in this experiment is essentially the same as described in a previous paper (2). The air flow rate through each train can be checked and adjusted independently by means of a bypass manifold attached to a flowmeter. Respiratory CO_2 from each flask was continuously swept out by a stream of CO_2-free air and absorbed in individual CO_2 traps. Flasks were shaken continuously in the light for six hours at 26° C. The respiratory CO_2 samples obtained during an experiment were precipitated as $BaCO_3$ and the radioactivity determined with a thin mica window GM counter (1.8 mg per cm^2). At the end of a 6 hour incubation period, sections were removed from the medium, rinsed with water, and homogenized with 25 ml ethyl alcohol. The radioactivity of the medium, alcohol extract of the tissues, and the alcohol insoluble residue were also determined by the conventional method in order to get necessary information on total C^{14} recovery. Total glucose uptake by stem tissues was calculated from the summation of radioactivity found in respiratory CO_2, alcohol insoluble residue and alcohol extract. In general, eight separate flasks (two stem samples from untreated plants and two stem samples each from plants treated with various amounts of 2,4-D) were carried out simultaneously in a single run. The weight of tissue used in each flask was adjusted to within 20 mg between samples in the first three experiments and to within 5 mg in the later runs. Unless otherwise indicated, the fresh weight of stem tissues used in each flask was between 1.7 and 1.8 gm.

Radiochemicals were purchased from Volk Radiochemical Co. and Nuclear Chicago Corp. All labeled substrates were adjusted to either 1 μc or 2 μc per mg of glucose prior to administration.

RESULTS AND DISCUSSION

Using C^{14} labeled glucose, the effect of 2,4-D on glucose utilization of bean stem tissues has been measured directly. It is assumed that the oxidation of radioactive carbon atoms from the absorbed glucose which subsequently was recovered in respiratory $C^{14}O_2$ was used to furnish the energy, while the radioactivity found in 80 % ethanol insoluble residue was presumably used in the synthesis of cell wall materials. As shown in table I, the percent of absorbed glucose-U-C^{14} recovered as $C^{14}O_2$ had an average value of 5.0 (± 0.4) from six individual runs in control stem tissues. In all cases, the radiochemical yields of respiratory $C^{14}O_2$ had greatly increased in 2,4-D treated

[1] Received October 26, 1950

[2] This work was supported by contract No. AT(45-1)-304 United States Atomic Energy Commission.

[3] Technical Paper No. 1274, Oregon Agricultural Experiment Station.

TABLE I

EFFECTS OF 2,4-D TREATMENT ON ABSORPTION AND METABOLISM OF GLUCOSE-U-C^{14} IN BEAN STEM TISSUES FROM PLANTS WHICH RECEIVED DIFFERENT DOSAGES OF 2,4-D

2,4-D Dosage μg/plant	Time of treatment days	Absorption/gm fresh tissue % Control	% Recovery of C^{14} from absorbed glucose-U-C^{14} in various fractions			Residue/CO_2
			Respiratory CO_2	EtOH Extract	EtOH Insoluble residue	
0(2)*	2	100	5.1	63.4	31.6	6.2
50(2)	2	140	9.1	57.7	33.2	3.6
100(2)	2	118	11.1	62.5	26.5	2.4
150(2)	2	118	16.4	51.0	32.6	2.0
0(4)	7	100	4.9	66.7	28.4	5.8
10(2)	7	222	8.6	46.1	45.3	5.3
20(2)	7	293	11.0	44.0	45.1	4.1
50(4)	7	375	15.1	48.5	36.3	2.4
100(2)	7	371	16.3	57.1	26.6	1.6
150(2)	7	362	17.2	54.5	28.3	1.6

* Denotes number of separate runs

stem tissues. The degree of increase was correlated with the dosage received by plants. The same effect may be obtained by using stem tissues either from plants receiving a higher dosage and harvested at a shorter time, or from plants receiving an appropriate lower dosage and harvested after a longer period. The percent radio-chemical yield of respiratory CO_2 from 2,4-D treated (50 μg/plant) stem tissues increased from 1.8 times (average value) by the 2nd day to a maximum by the 7th day which varied from 3.0 to 3.2 times the corresponding rate of untreated tissues. These results are in good agreement with the work reported by Smith (7) using the manometric technique. The influence of 2,4-D on the incorporation of isotopic carbon from the absorbed glucose into alcohol insoluble residue was not observed from samples harvested 2 days after treatment. A significant increase in the rate of such incorporation was noted, however, in the 7th day samples receiving 10, 20, and 50 μg/plant treatment, and no effect was observed at the 100 and 150 μg/plant application. This increase was not dependent upon the dosage of treatments. It is of interest to note that the ratio of radiochemical recoveries between ethanol insoluble residue and respiratory CO_2 decreased from an average value of 6.0 for the untreated tissues to 1.8 for 2,4-D tissue from plants receiving 150 μg per plant dosage. Figure 1 shows a plot of 2,4-D dosages and the percent decrease in residue/CO_2 ratio (average values from G-U-C^{14}, G-1-C^{14} and G-6-C^{14} experiments), and a plot of dosages and the increase of C_6/C_1. The results indicate a direct relationship between 2,4-D dosages and the influence on glucose utilization and the catabolic pathways in the stem tissue of bean plants.

Uptake of glucose by 2,4-D treated stem tissues was greatly increased. This observed increase was much less in the 2nd day samples than in the 7th day samples. Dosage in excess of 50 μg per plant did not show an additional increasing effect; however, at a lower rate the increase of uptake per 1 gm fresh tis-

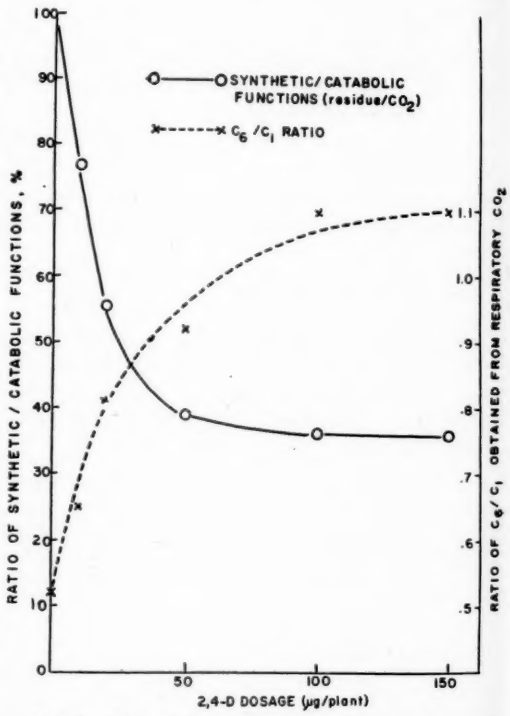


FIG. 1. Plot of 2,4-D dosages and percent decrease in residue/CO_2 ratio, and plot of dosages and increase of C_6/C_1.

sue seemed to be correlated with the amount of 2,4-D applied. Humphreys and Dugger (5) found that 2,4-D treatment inhibited the uptake of glucose in corn roots. This evidence would strongly suggest that 2,4-D may have a different function of modifying the biochemical processes in different plant tissues as well as from different plant species.

Table II summarizes the results of radiochemical yields in the respiratory CO_2, ethanol insoluble residue, and ethanol extract from G-1-C^{14} and G-6-C^{14} experiments. The percent recoveries of these fractions is based upon the glucose uptake by the stem tissues rather than by the method customarily used which is based on the amount of glucose added to the flask. From these data, the C_6/C_1 ratios were calculated. The CO_2 yields from the absorbed G-1-C^{14} and G-6-C^{14} in untreated stem tissues were very consistent between six individual runs. It was 2.7 ± 0.3 % for G-6-C^{14} and 5.2 ± 0.8 % for G-1-C^{14}. The average value of C_6/C_1 ratio from this result was 0.52(±0.10) (range from 0.37 to 0.67). These data are in good agreement with the results found in stem tissues from other plant species (1,3). The percent $C^{14}O_2$ yields from both G-1-C^{14} and G-6-C^{14} were greatly increased in 2,4-D treated stem tissues. The degree of influence is correlated with 2,4-D dosage up to 100 μg per plant. Because the percentage change of $C^{14}O_2$ yield from G-6-C^{14} was greater than that from G-1-C^{14}, the C_6/C_1 values calculated from these results show a steady increase to unity with increasing of 2,4-D dosages. This evidence was in direct contrast with the findings reported by Humphreys and Dugger (4) using the root tissues of pea, corn, and oat seedlings. An opposite influence which was caused by the 2,4-D treatment on the pathways of glucose catabolism in different organs is difficult to explain.

In untreated stem tissue the participation of C-1 carbon from applied glucose in the synthesis of cell wall materials was 26.8 ± 4.0 % and of C-6 carbon was 30.2 ± 2.7 %. The average value of such participation from the whole glucose molecule was 28.4 %. 2,4-D treatment at low dosages (10-20 μg per plant) caused a slightly increased participation of C-1 and C-6 in synthetic function, which was in agreement with the results from glucose-U-C^{14} experiments. At higher dosage (50-150 μg per plant) the influence of 2,4-D in this respect was uncertain.

It has been pointed out that the carbohydrate metabolism in higher plants undergoes a qualitative change as the plants develop (3). In general, the glucose catabolism is exclusively via the E.M.P. pathway in young and meristematic tissues. As the growth and differentiation occurred, the contribution of the pentose cycle pathway on glucose breakdown increased (3). If we again consider the direct measurement of percent radiochemical yields of respiratory $C^{14}O_2$ and the ethanol insoluble residue as an approximation of the catabolic and synthetic functions of glucose, the ratio calculated from these results could be used for a description of glucose utilization. The ratios from these experiments were 5.2, 11.2, and 6.0 for C-1, C-6, and C-U carbons of glucose, respectively, in the untreated stem tissue. This dif-

TABLE II

EFFECTS OF 2,4-D TREATMENT ON ABSORPTION AND METABOLISM OF GLUCOSE-1-C^{14} AND GLUCOSE-6-C^{14} IN BEAN STEM TISSUES FROM PLANTS WHICH RECEIVED DIFFERENT DOSAGE OF 2,4-D

SUBSTRATE	μG/PLANT	TIME OF TREATMENT, DAYS	ABSORPTION/GM FRESH TISSUE % CONTROL	% RECOVERY OF C^{14} FROM ABSORBED GLUCOSE IN VARIOUS FRACTIONS			RESIDUE/ CO_2
				RESPIRATORY CO_2	ETOH EXTRACT	ETOH INSOLUBLE RESIDUE	
G-1-C^{14}	0(6)*	7	100	5.2 ± 0.8	68.5 ± 4.9	26.8 ± 4.0	5.2
	10(4)	7	239 ± 32	8.6 ± 1.6	57.4 ± 3.3	34.1 ± 2.8	4.0
	20(4)	7	304 ± 42	9.0 ± 3.3	58.1 ± 2.5	33.0 ± 1.6	3.7
	50(6)	7	269 ± 25	10.0 ± 1.9	64.2 ± 3.6	25.8 ± 1.9	2.6
	100(1)	7	338	10.8	56.3	32.9	3.0
	150(1)	7	303	10.3	57.9	31.8	3.1
G-6-C^{14}	0(6)	7	100	2.7 ± 0.3	67.1 ± 2.8	30.2 ± 2.7	11.2
	10(4)	7	233 ± 27	5.6 ± 1.1	55.8 ± 3.8	38.6 ± 3.0	6.9
	20(4)	7	296 ± 25	7.3 ± 1.1	58.4 ± 4.2	33.7 ± 3.7	4.6
	50(6)	7	278 ± 29	9.2 ± 1.2	62.2 ± 2.6	28.6 ± 3.4	3.1
	100(1)	7	264	11.9	60.9	27.2	2.3
	150(1)	7	316	11.4	56.8	31.8	2.8
C_6/C_1	0			0.52	0.98	1.13	
	10			0.65	0.97	1.13	
	20			0.81	1.00	1.02	
	50			0.92	0.97	1.11	
	100			1.10	1.08	0.83	
	150			1.10	0.98	1.00	

* Denotes number of separate runs

ference found between C-1 and C-6 could be due to the participation of pentose cycle pathway. When the participation of E.M.P. pathway on glucose catabolism was increased in the stem tissue of bean plants as a result of 2,4-D treatment, the ratio between synthetic function and catabolic function of glucose from G-1-C^{14} and G-6-C^{14} experiments should change accordingly. No difference, either in the yield of respired $C^{14}O_2$, or in the ratio of synthetic/catabolic functions should be found between C-1 and C-6 carbons when the E.M.P. pathway is operating exclusively. The experimental results as shown in tables I and II indicate this was the case. The values are 2.6, 3.1, and 2.4 for C-1, C-6, and C-U, respectively, in the 2,4-D treated stem tissues (7 days after 50 μg/plant treatment).

No significant influence as a result of 2,4-D treatment was noted between C_6/C_1 values calculated from the percent radiochemical yields either in the ethanol insoluble residue or in the ethanol extract, indicating that the synthetic pathways for cellular constituents are not affected.

Summary

The effect of 2,4-D upon the absorption and metabolism of glucose in bean stem tissues was studied. After a 2 day treatment period, the uptake of labeled glucose by bean stem tissue was the same as the control; after 7 days, however, there was a threefold increase over the control. The percent radiochemical yield of respiratory CO_2 and the glucose utilization as expressed by the ratio of synthetic function/catabolic function from either C^{14} labeled glucose were changed in 2,4-D treated stem tissues. These changes were correlated with the dosage received by the plant.

The yields of $C^{14}O_2$ from glucose-1-C^{14} and glucose-6-C^{14} in short time experiments were used to evaluate the effect of 2,4-D on the participation of the direct oxidative pathway in glucose catabolism. Treatment with various amounts of 2,4-D caused a relative increase in the amount of glucose catabolized via the glycolytic sequences.

Evidence was obtained to indicate that the synthetic pathways for cellular constituents in the stem tissue are not affected by 2,4-D.

Literature Cited

1. Beevers, H. and M. Gibbs. 1954. The direct oxidation pathways in plant respiration. Plant Physiol. 29: 322–324.
2. Fang, S. C., Patricia Theisen, and J. S. Butts. 1959. Metabolic studies of applied indoleacetic acid-1-C^{14} in plant tissues as affected by light and 2,4-D treatment. Plant Physiol. 34: 26–32.
3. Gibbs, M. and H. Reevers. 1955. Glucose dissimilation in the higher plant. Effect of age of tissue. Plant Physiol. 30: 343–347.
4. Humphreys, T. E. and W. M. Dugger, Jr. 1957. The effect of 2,4-dichlorophenoxyacetic acid on pathways of glucose catabolism in higher plants. Plant Physiol. 32: 136–140.
5. Humphreys, T. E. and W. M. Dugger, Jr. 1959. Effect of 2,4-dichlorophenoxyacetic acid and 2,4-dinitrophenol on the uptake and metabolism of exogenous substrates by corn roots. Plant Physiol. 34: 112–116.
6. Kelly, Sally M. and G. S. Avery, Jr. 1949. The effect of 2,4-dichlorophenoxyacetic acid and other physiologically active substances on respiration. Amer. Jour. Bot. 36: 421–426.
7. Smith, F. G. 1948. The effect of 2,4-dichlorophenoxyacetic acid on the respiratory metabolism of bean stem tissue. Plant Physiol. 23: 70–83.

INSTRUCTIONS FOR CONTRIBUTORS TO PLANT PHYSIOLOGY

I. Writing Manuscript

A. Style In general, the style of an article will reflect the author's preference and habits of writing. The Editor may suggest improvements, but will not insist on rigid conformity to conventions at the expense of an author's individuality. Scientific writing, however, should meet accepted standards of grammar, syntax, and diction.

Strive for specific and concise expression. Use active verbs. Avoid complicated sentence structure. Write in a straightforward manner. Use care in preparing the original manuscript to obviate subsequent delays in publication because of the need for manuscript revision.

B. Length Beyond ten pages (including tables, figures, and literature citations) authors will be billed $20 per page for overrun. Approximately four double spaced typewritten pages (pica type) equal one page of printed type.

C. Footnote References These should be avoided in the body of the text, but if a footnote must be used, it should be numbered with an Arabic numeral and typed at the bottom of the page on which reference to the footnote is made.

D. Acknowledgements Do not include acknowledgements in a footnote; they belong under their own heading following the SUMMARY and preceding LITERATURE CITED.

E. Punctuation

1. Periods and Commas These are the most often used and misused punctuation marks; the period is not used enough, the comma too often. Avoid complicated sentences which require many commas, colons, or semicolons. Break long sentences into brief, concise sentences which will be short and easily understood.

2. Quotation Marks Avoid the urge to use excessive quotation marks. Often a word is used in a sense slightly different from its ordinary meaning. This does not necessarily warrant the use of quotation marks. In case a term is used in an unconventional way and the ambiguity might lead to misunderstanding, use quotation marks only when the unfamiliar meaning is first explained.

3. Hyphens Use sparingly.

F. Subtopics Use this order and style to number subtopics:

I.	I)
A.	A)
1.	1)
a.	a)

G. Scientific Names The complete botanical name (genus, species, and authority for the binomial) of all experimental plants should be included, preferably under MATERIALS AND METHODS. Refer to the most recent International Code of Botanical Nomenclature for rules governing the use of scientific names.

H. Abbreviations In place of certain unwieldy chemical names abbreviations may be used as a convenience. The writing, however, must remain readily comprehensible and unambigious. For the most part only widely used abbreviations should be employed (e.g.: ATP, RNA, TPN). Standard chemical symbols may be used without definition: Ca, NaOH. All others should be defined when they first appear. Example: The reaction was thought to involve flavin adenine dinucleotide (FAD). If your article uses several abbreviated forms it is permissible to define them all in a single footnote where the first abbreviation is introduced.

In no case should the number of nonstandard abbreviations be so large that the reader is forced to learn a special vocabulary in order to read your article easily. Abbreviations should not frustrate the reader; they should be used for his convenience, not the author's convenience.

Avoid abbreviations in title and summary. Because these parts frequently are translated into foreign languages, undefined abbreviations may be confusing.

I. Chemical Formulas It is expensive (sometimes impossible) to set formulas in type. Also there is danger of introducing errors. Submit structural formulas or involved reaction sequences as clearly executed India ink drawings which can be reproduced photographically. Indicate where these are to be inserted into the text. Alternatively a complex reaction diagram or flow sheet may be treated as a regular figure, given a number, and supplied with a legend.

II. Preparing Typescript

Carefully type copy in accordance with instructions. Give special attention to the form used by the Journal for tables, figure legends, and literature citations. Consult recent editions of PLANT PHYSIOLOGY for proper placement of main headings, paragraph headings, and subheadings, and for the use of abbreviations.

A. Spacing Double space everything, including tables, legends, literature cited, and footnotes. It is essential that all copy be double spaced to allow room for the extensive proofmarking which must

be done (even to a perfect typescript) in the editorial office so the printer will know precisely how to set your copy in type.

Begin typing 1/3 of the way down the first page; leave liberal side margins.

Each table, each set of figures, legends, and literature citations should be doubled spaced on a separate piece of paper; these items are given by the printer to different people for setting in type sizes different from that in the body of the manuscript.

B. ORDER Arrange manuscript copy in this order:

1. Name and address for mailing proofs
2. Title of article
3. Author's name and institution
4. Text
5. Summary
6. Acknowledgements
7. Literature cited
8. Tables
9. Legends for figures
10. Figures

C. PAGING Number all pages consecutively in upper right hand corner. Never use a letter such as 2a, 5D, for paging; such pages are easily lost during processing.

D. PAGE ONE Do not use a title page.

1. Name and Address On page 1 above the title of the article, put the name and address of the person to whom editorial correspondence (including galley proof and reprint order form) is to be sent. The name and address of the institution where the work was done follow the name of the author directly below the title.

2. Footnotes One or more footnotes are included at the bottom of the first page. The first footnote (indicated by superscript 1 after the title) gives the date the manuscript was received. The second footnote credits the source of financial support (if any). Additional footnotes give the present address of the author, if different from the address in the heading, and indicate the experiment station or institution paper number. Normally these are the only footnotes used.

E. NUMBERS

1. Write out all numbers, cardinal and ordinal, from one through ten. Use figures for all numbers larger than ten. Exceptions:

a. When using precise measurements such as 5 per cent, 6 mm, 8-hour cycle, figure 1. In nonprecise usages spell numbers out: about two weeks, six or eight degrees.

b. In a series of related numerical expressions treat all alike even though violating the general rule: 2 leaves, 8 leaves, and 12 leaves.

c. When a single expression has consecutive numbers, use both figures and spelled out numerals: two 6-min exposures.

d. Spell out ALL numbers or fractions which begin a sentence. If this is awkward, rephrase the sentence to avoid beginning with a numeral.

e. Do not use a hyphen to replace the preposition "to" between numerals: 13 to 22 min, three to ten degrees. Exceptions: tables, figures, graphs.

F. FRACTIONS Write out and hyphenate nonprecise simple fractions: two-thirds, *but* half strength. Exceptions: tables, figures, graphs, legends, fractions in parentheses. Often it is desirable to use decimals instead of fractions. For values less than one, insert a zero before the decimal point: 0.7 g *not* .7 g.

G. LITERATURE CITED This section of a manuscript requires extensive proofmarking even though copy is correct in every detail. Typing errors are easily made, but difficult to detect, so exercise special care in preparing and checking the bibliography. Since they are set in a typeform different from that used for the body of the manuscript, literature citations should be *double spaced on a separate piece of paper.* Consult the most recent issue of PLANT PHYSIOLOGY for Journal style. Note the limited use of capitals.

Check all citations (such as title, volume number, pages, and date) for completeness. Check foreign titles for diaresis and other markings. Make certain that all citations are referred to in the text.

List citations in alphabetical order (not in order of reference), then number them and indicate citations in the body of the text by Arabic numerals enclosed in parenthesis.

Unpublished data, personal communications, and articles in preparation are not acceptable as literature citations, but should be referred to parenthetically in the text. Articles that are in press may be so designated in LITERATURE CITED. An article is not properly referred to as "in press" unless it has been *accepted* for publication. The journal in which an in press article will appear should be included in the literature citation.

1. Authorship List family name of first or sole author before listing initials or given name. Thereafter write names and initials (or given name) of all coauthors in their natural order. Use initials for a man's given name; write out a woman's given name: Reeves, A.C., R. D. Jones, and Helen Smith.

2. Date The year belongs immediately after the author's name.

H. HYPHENS Avoid dividing a word at the end of a line. Such division increases the likelihood of a printer's error.

I. UNDERLINING In general, do not underscore anything. When copy is marked for the printer,

headings, scientific names, etc., will be underscored in the Editor's office. Exception: *rarely* a word or phrase may be underlined for appropriate emphasis.

J. SPELLING Draw a box around unusual spelling to indicate that the word has been spelled correctly.

III. PREPARING TABLES

Authors should set up their tables in a form consistent with recent issues of PLANT PHYSIOLOGY. Number tables consecutively in Roman numerals and place the author's name on the back of each page.

A. COPYFITTING Tables should be preplanned to fit one or two printed columns (3 inches or 6⅛ inches wide, respectively) and not to exceed 8½ inches in height, including heading, body, and footnotes. Tables may not be rotated 90°.

On the average, about 52 typewriter spaces (excluding the new executive style of typewriter) fill one 3-inch line of type of the size normally used in tables; ten double spaced typewritten lines require 1 inch of the available vertical printed space.

B. NUMERALS Check tabular data as well as numerical values reported in the text for the proper number of significant figures. Frequently authors report more digits than are significant. Use three dots (*not* a dash) to indicate a blank in a table: . . . For decimals smaller than one, insert a zero before the decimal point: 0.349.

C. FOOTNOTE DESIGNATIONS Refer to footnotes by the asterisk, double asterisk, triple asterisk, dagger, double dagger, and section, in this sequence, as needed: *, **, ***, †, ††, ‡. *Place all explanatory material in footnotes* rather than in the heading or columnar headings, both of which should be as *brief as possible* without sacrificing clarity.

D. HEADINGS AND FOOTNOTES Wherever possible, omit unnecessary articles such as "the", "a", in headings and notes. Also avoid bulky prepositional phrases.

IV. ILLUSTRATIONS

A. PRINTS FOR REVIEW For manuscript review it is satisfactory to furnish illustrations which have been reproduced by such economical copy methods as Verifax and blue print. Thus prints which will be used for publication need not be sent through the mail repeatedly. *Do not send original drawings or photos* at any time.

B. PRINTS FOR REPRODUCTION After your manuscript has been accepted for publication you must submit good quality glossy prints (glossies) for making electrotypes.

1. Kinds of Illustrations Figures are of two kinds, line drawings and halftones. Line drawings are just that—line drawings usually done with India ink on white paper. Halftones, in contrast to line drawings, are not just black and white, but they also are shaded from black to white. Because halftones are photographed through a screen some detail is lost in reproduction.

2. Special Treatment of Photographs For illustrations in which it is especially important to bring out all the detail present in the original photograph special processing will be undertaken. Should your paper contain electron-micrographs, radioautographs, or other material whose reproduction should be deemed critical, call the editor's attention to the need for special processing.

3. Good Illustrations Plan in advance for clear, legible illustrations. Line drawings should be intensely black on white; halftones should have good contrast and be in sharp focus throughout. No glossy should contain blemishes. Do not use paper clips on photos; grooves, gouges, and creases show when reproduced.

Generally the appearance of a graph is improved if the top and right sides are boxed in by lines of the same weight as the abscissa and ordinate.

Guided lettering is preferable to hand lettering or typing.

4. Size Although the Editor determines the final printed size of an illustration, an author should plan for maximum reduction. If symbols, such as triangles, circles, and squares (or letters and numerals) are not sufficiently large to begin with, they may become mere blurs when reproduced. Any letter smaller than 1 mm may be illegible. Exceedingly large symbols, thick lines, or heavy numerals may become unsightly when reduced.

Figures are reproduced the same size (x 1) or they are reduced. (Enlarging magnifies defects.) Illustrations are fitted either into 3 inch or 6⅛ inch column widths. Although occasionally an illustration must be 8½ inches high, this is undesirable. One should allow space at the bottom for the figure legend. It may help you to sketch a rough onion skin overlay of a typical Journal page layout for estimating final reduced size of an illustration. Final size, however, cannot be estimated until after illustrations are properly grouped and mounted.

5. Grouping One large illustration is cheaper to reproduce than three to six small ones, so *as far as possible group all figures.* Mount similar figures close together. Place halftone and line drawings in separate groups.

Limit the area of each photograph to that which is necessary to show pertinent details. Trim off irrelevant portions and minimize blank space.

6. Mounting Mount all illustrations with rubber cement on white cardboard or stiff white paper. Total size should not exceed 9 x 11

inches. Anything larger than an ordinary file folder is easily mutilated; it also requires special packaging and handling.

7. Identification Place the author's name on the back of each figure and legend. Place the figure number on the back of each figure. Identification insures against misplacement.

V. Submitting Manuscripts

Send the original copy of the manuscript, the first carbon, and two copies of all illustrative material to the Editor, Dr. Allan H. Brown; Department of Botany; University of Minnesota; Minneapolis 14, Minn. Retain a carbon copy of your manuscript and original photographs or drawings to insure against loss.

PLANT PHYSIOLOGY does not accept a manuscript which has not been submitted directly by an author. If your manuscript is submitted by someone other than one of the authors, send a letter to this office stating that you wish to publish that particular manuscript in PLANT PHYSIOLOGY. Your manuscript cannot be processed until this letter is received. This precaution protects you as well as the Journal.

VI. Processing Manuscript

A. Review The manuscript will be sent usually to two reviewers familiar with that field of research covered by the paper. Each reviewer evaluates the manuscript, suggests improvements, and recommends accepting or rejecting the paper. Usually two months suffice for review and decision. When there is marked disagreement between reviewers, the Editor may appoint a referee (often a member of the Editorial Board) to evaluate the manuscript in light of the reviews; the referee recommends either for or against acceptance.

B. Revision Manuscripts returned to the author for revision should be sent back to the Editor within two months; otherwise the paper will be published under the date of receipt of the revised manuscript and not the original date.

Retype each page on which there are extensive changes.

C. Galley Proof This will be sent to the person designated on page 1 above the title. (See II D 1).

1. Returning Proofs Return only one set of galley proofs to Editor. Keep any other set for reference. Be certain to notify Editor promptly of change of address, so galleys can be sent to the correct address.

2. Charges for Errors You will not be charged for correcting printer's errors, BUT you will be charged at cost for all changes or corrections in galley which are your fault. Here are two ways to minimize charges:

a. Proofread Manuscript Check ALL references carefully BEFORE submitting manuscript to Editor. Corrections in typing are easy to make, whereas corrections in galley or page proof are expensive and sometimes difficult to make.

b. Corrections Where a correction is optional remember that a change made near the beginning of a paragraph can necessitate resetting every subsequent line to the end of the paragraph. The printer charges for each line he must reset to correct an error, so weigh the cost against the importance of the change you propose.

D. Delays In general, a manuscript should be reviewed and returned to you with the Editor's decision within two months. After you send a revised manuscript to the Editor allow 2½ months for galleys to be sent you for correcting. Should you find that processing your manuscript has taken an unusually long time at either stage, it would be well to inquire of the Editor as to the cause of the delay. (This is a precaution against unrecognized loss of manuscripts in the mail—a rare event, but it happens.)

E. Errata If necessary, an errata will be published for each volume. Authors therefore are requested to call the attention of the Editor to any significant errors in their published manuscripts.

e
T
r
t.

L
g
o-
ns
ıd

p-
ar
si-
he
es
an
n-

be
r's
re-
ths
uld
nas
it
the
nst
—a

ub-
are
any
pts.